Universidade Vigo

Departamento de Ecoloxía e Bioloxía Animal

Caracterización taxonómica y ecología de especies crípticas o pseudocrípticas de dinoflagelados nocivos

Santiago Fraga Rivas

Memoria de Tese de Doutoramento para optar ao título de Doutor pola Universidade de Vigo

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Memoria presentada por Santiago Fraga Rivas para optar ao título de Doutor pola Universidade de Vigo

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En los comienzos de mi carrera me dediqué al monitoreo de las especies tóxicas y a la ecología. En aquella época de autodidacta, clasificaba fitoplancton con la idea de que las especies eran algo perfectamente definido. En 1983 participé en el "Phytoplankton Course for Experienced Participants" organizado por la Universidad de Oslo en Drobak, donde tuve la gran suerte de tener como profesores a Grethe Hasle, Jahn Throndsen, Karl Tangen, Berit Heimdal y a Karen Steidinger y como compañeros, entre otros, a Carmelo Tomas, Øjvind Moestrup, Gustaaf Hallegraef y Maria Faust con quienes he seguido en contacto y de quienes he aprendido mucho. En aquel curso, aprendí de los grandes gurús a raramente decir sí o no, sino «could be». Participar a continuación en un proyecto con Karen Steidinger fue otra gran suerte que he tenido y fue ella quien me abrió los ojos cuando observando un *Prorocentrum* comentó que podría tratarse de una nueva especie. Barrie Dale me invitó a participar en dos talleres em Sherkin Island, una agreste isla en el suroeste de Irlanda, donde durante una semana tuve la oportunidad de observar muestras con Enrique Balech a mi lado y de tener largas conversaciones con él de las que salió una profunda amistad que me permitió seguir aprendiendo de él, no solo ciencia, a través de sus inolvidables cartas escritas con una máquina de escribir prehistórica y en papel avión. También en esos talleres hubo tiempo de conversar muchas horas con Malte Elbrachter, Karen y Gustaaf, además de compartir unas cuantas pintas de Guinnes en las noches del Jolly Roger.

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«On ne fait jamais attention *à* ce qui *a* été fait; on ne voit que ce qui *reste à faire*»

Marie *Curie*

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INTRODUCCIÓN

NECESIDAD DE TAXONOMÍA

La clasificación siempre ha sido una de la preocupaciones fundamentales de la ciencia pues los hechos y los objetos deben disponerse de una forma ordenada antes de que se puedan descubrir sus principios unificadores. El objeto de la taxonomía es pues agrupar los objetos en taxones naturales (Sokal, 1966). El establecimiento de una taxonomía estable es particularmente importante para cualquier campo de la ciencia que se base en medidas exactas de la biodiversidad, incluyendo la ecología y la conservación (Fujita *et al.*, 2012). Las investigaciones sobre biodiversidad y ecología descansan sobre una identificación segura de las especies y en conteos reproducibles, pero esto no siempre se consigue (Boenigk *et al.*, 2012). Esta identificación es especialmente importante cuando se trata de especies tóxicas, ya que no siempre es fácil distinguir morfológicamente una especie tóxica de la que no lo es, haciendo de las tareas de monitoreo una labor a veces muy imprecisa. Para tratar de resolver estos problemas está la taxonomía, que es la disciplina biológica que describe, clasifica y nombra especies y otros taxa, tanto actuales como extintos (Padial *et al.*, 2010).

El reto científico para el futuro de la taxonomía es abordar cómo deben caracterizarse las especies. Hay que tener en cuenta que las dificultades no están sólo en la identificación de una especie, sino previamente en su delimitación.

Los principales objetivos de la taxonomía sistemática son descubrir y describir especies y determinar las relaciones filogenéticas entre esas especies (Wiens, 2007).

¿Existen las especies?

Esta es la pregunta clave. A lo largo de la toda la historia de la humanidad se han distinguido «especies» sin tener claro si éstas realmente existen como tal, o no. Ya en la Biblia, relatando el diluvio y el arca de Noé, se decía: *Y de todo lo que vive, de toda carne, dos de cada especie meterás en el arca, para que tengan vida contigo; macho y hembra serán. De las aves según su especie, y de las bestias según su especie, de todo reptil de la tierra según su especie, dos de cada especie entrarán contigo, para que tengan vida*. (Génesis 6:19; 6:20). Allí, se da por sabido que las especies se diferencian morfológicamente pero, al señalar que se escojan dos individuos, un macho y una hembra, se considera implícitamente que una especie es un grupo de individuos y que la reproducción sexual es parte de su entidad. Otro ejemplo de que la idea de especie es fuerte en otras civilizaciones, se da en la tribu Ketengban de Nueva Guinea, cuyos

miembros dependen de la caza y la recolección para sobrevivir. Los miembros de esta tribu dan nombres diferentes a 169 especies de aves, de los que a 143 de ellos se le ha conseguido encontrar su correspondencia con nombres latinos de especies descritas científicamente (Diamond & Bishop, 1999). Estos nativos de Nueva Guinea distinguen las aves, no por pequeños detalles morfológicos como los usados tradicionalmente por los ornitólogos, sino por un conjunto de caracteres tales como el canto, la silueta, la postura, el comportamiento y la apariencia general. Aquí los finos caracteres morfológicos son de poca utilidad para identificar un ave en la espesura de los bosques tropicales sin la utilización de unos prismáticos (Diamond & Bishop, 1999). Aunque los métodos utilizados por los miembros de la tribu Ketengban para diferenciar especies sean diferentes que los usados por los ornitólogos científicos, los resultados son sorprendentemente muy parecidos e incluyen la identificación de pares de algunas especies muy difíciles de distinguir por ornitólogos experimentados. Esta coincidencia en los resultados entre culturas y métodos tan diferentes hace pensar que las especies son entes reales. Sin embargo, por encima del nivel de especie, los miembros de la tribu Ketengban solo distinguen aves, murciélagos, mamíferos no murciélagos, serpientes, lagartos y ranas. No tienen nombres para grupos como loros o aves rapaces (Diamond & Bishop, 1999) lo que muestra la fuerte entidad que tiene la especie para estas personas en comparación con otros niveles taxonómicos superiores en la jerarquía hoy utilizada por la ciencia.

Los estudios etnobiológicos revelan principios universales que reflejan la capacidad de la mente para captar y organizar en taxonomías a aquellas especies que, desde el punto de su percepción, son importantes (Atran *et al.*, 1999). Berlin (1992) observa regularidades en la clasificación y denominación de las plantas y los animales entre los pueblos de sociedades primitivas analfabetas, regularidades que persisten más allá de entornos locales, culturas, sociedades y lenguas. Berlin (1992) sostiene que estos patrones se pueden explicar mejor por la similitud, en gran parte inconsciente, en la percepción de los seres humanos de las afinidades naturales entre grupos de plantas y animales. Las personas reconocen y nombran grupos de organismos independientemente de su utilidad real o potencial o de su significado simbólico en la sociedad humana.

El concepto de especie ha sido muy discutido desde el punto de vista teórico a lo largo de toda la historia aunque, sin embargo, a nivel práctico, implícitamente se ha utilizado el concepto morfológico que, como se discute más adelante, define una especie como una comunidad, o un número de comunidades relacionadas, cuyos caracteres morfológicos distintivos están, en la opinión de un sistemático competente, suficientemente definidos como para denominarla o denominarlas con un nombre específico (Regan, 1926: 75 citado por (Mayden, 1997)), o como irónicamente dice Sokal (1966) «*a species is whatever a competent taxonomist decides to call a species*». Es decir, algo

fundamentalmente subjetivo y a menudo resultado de decisiones arbitrarias de taxónomos convencionales.

Yendo a la historia antigua de la Ciencia, Platón consideraba la especie como algo inmutable y es ésta la idea que a nivel popular todavía se usa hoy en día: nadie duda de que una vaca pertenece a una especie diferente a la de una de oveja, y de que un pino y un roble son especies diferentes. Sin embargo, ya Aristóteles cuestionó la idea platónica aunque injustamente se le considere, junto con Platón, uno de los autores del concepto tipológico de especie (Marcos, 2009) según el cual un individuo transmite su forma a otras generaciones de un modo inmutable. El primer problema que se plantea para definir qué es una especie es resolver previamente si ésta existe o no. En la antigüedad la explicación era la esencialista, representada por Platón, Aristóteles y San Agustín, quienes afirmaban la existencia real de los universales que constituían la más auténtica realidad, que los individuales no difieren en esencia y que los géneros y las especies existen realmente. Por el contrario, al final de la Edad Media esta visión realista o esencialista se enfrentó con la nominalista. Los nominalistas como William of Ockham (Siglo XIV) afirman que los universales son exclusivamente creaciones de la mente humana: son simplemente términos o nombres (razón por la que a esta doctrina se la conoce como terminismo o nominalismo). Para ellos, el género y la especie no tienen existencia real. La verdadera sustancia es el individuo, como, en cierto modo ya había reconocido Aristóteles (Marías & Laín Entralgo, 1964, Llorente Bousquets & Michán Aguirre, 2000). Esta polémica ha perdurado más o menos encubierta hasta ahora y sigue totalmente vigente aunque la mayoría de los biólogos modernos están de acuerdo en que la especies son entidades reales, y que son las unidades fundamentales de la taxonomía, la biodiversidad y la evolución.

DESARROLLO HISTÓRICO DE LA TAXONOMÍA

Los comienzos de la taxonomía, tal como hoy en día se entiende, comienzan en el siglo XVII con John Ray que mantuvo en su *Historia Plantarum* que las plantas no pueden transmitir a sus descendientes características adquiridas accidentalmente, y sobre todo con Linneo en el siglo XVIII, quien a través de sus libros *Systema Naturae* y *Species Plantarum*, sentó las bases de la taxonomía actual reflejada en los vigentes códigos de nomenclatura botánica y zoológica. En esta época la ciencia estaba dominada por la concepción religiosa de tal modo que las especies habían sido creadas por Dios y transmitían sus características a su descendencia. El hecho de que algunos híbridos como la mula, fuesen estériles era un mecanismo planificado por el creador para proteger su integridad. Las variaciones morfológicas dentro de una especie eran simples aberraciones del tipo original creado por Dios, lo cual da lugar al concepto tipológico de especie.

Con la publicación de *On the Origin of the Species* de Darwin en 1859, se dio un salto cualitativo en el concepto teórico de especie al dejar ésta de ser considerada algo inmutable, y pasar a ser considerada como algo en continua evolución. Sin embargo, Darwin, en contra de lo que se podría esperar, no entra a fondo en el concepto de especie ya que él simplemente la considera como un nivel más dentro de la jerarquía taxonómica entre el género y la variedad. A pesar del título de su obra, Darwin trata más sobre los cambios en una especie que sobre su origen. Él no habla solamente de su evolución sino de su división en otras especies, hasta tal punto, que la única figura publicada en *The Origin of Species* ilustra esa división (Fig 1).

Darwin no considera las especies como algo real cuando dice: *From these remarks it will be seen that I look at the term of species, as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms* (Darwin, 1859) (pág. 70). Darwin discute cómo distinguir una especie de sus variedades basándose fundamentalmente en plantas y analizando floras de diversos países pero no entra en su definición conceptual. Aunque Darwin dudara de la existencia de las

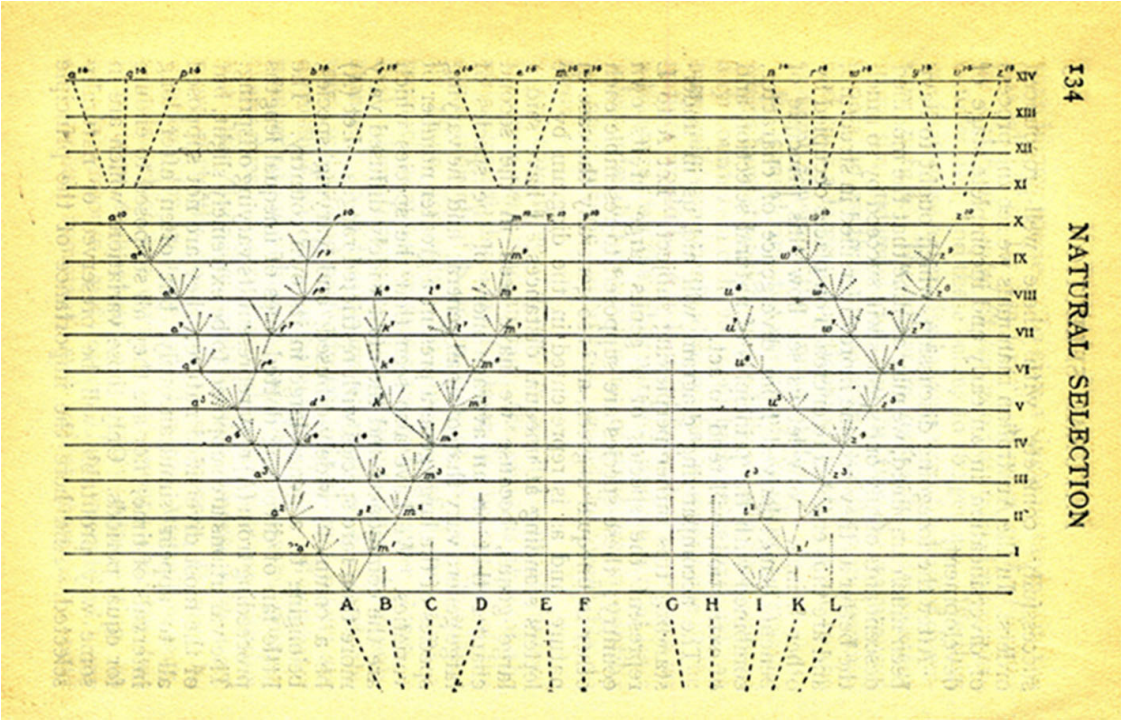

Figura 1. Reproducción de la única figura publicada en el «On The Origin Of Species by Means Of Natural Selection» de Charles Darwin 1859 (Edición de Collins' Clear-Type Press)

especies como unidades básicas de la naturaleza, su teoría de la evolución tuvo profundas implicaciones en futuras interpretaciones de las especies. Como contraste a la idea religiosa del tipo, él consideraba la esterilidad de los híbridos como un producto secundario de la selección natural y no como un mecanismo para proteger su integridad. Del mismo modo consideraba que las variaciones intraespecíficas eran una parte integral del proceso de selección natural que da lugar a la evolución de las especies, y no como imperfecciones del tipo original. En el pensamiento de Darwin hay que diferenciar entre especie como un taxón, de especie como categoría. El taxón especie es un grupo de organismos que comparten ciertas características, por ejemplo, *Homo sapiens* o *Canis familiaris*. Sin embargo, a la categoría de especie, considerada como el conjunto de todos los taxones específicos, se la considera como un nivel más dentro de la jerarquía linneana (Ereshefsky, 2010). Cuando Darwin dice: *«I mean by species, those collections of individuals, which have commonly been so designated by naturalists. Everyone loosely understands what is meant when one speaks of the cabbage, Radish & sea-kale as species; or of the Broccoli, & cauliflowers as varieties»* (Darwin, 1975, pág. 98) de lo que duda, no es del taxón especie, sino de la existencia de la especie como categoría.

A partir de ese momento arreciaron las discusiones teóricas y académicas sobre qué es una especie, pero sin embargo, a efectos prácticos se siguió el método de Linneo y se continuaron, y se continúan, describiendo especies sin tener para nada en cuenta los principios de la evolución descritos por Darwin actuando como si las especies fuesen algo perfectamente delimitado e inmutable. Los códigos de nomenclatura se siguen basando en el concepto tipológico de especie y nacieron como fruto de la necesidad de poner unas normas para el uso del sistema binomial de nomenclatura establecido por Linneo. Los dinoflagelados, que son el tema de esta tesis, han sido tradicionalmente descritos utilizando los códigos de nomenclatura zoológico o botánico de acuerdo con el gusto de los autores o las exigencias de la revista en donde se publiquen las descripciones de nuevas especies, como ocurre con las revistas ficológicas que exigían el uso del código botánico. El vigente *International Code of Zoological Nomenclature* con validez desde el 1 de enero de 2000 dice tajantemente entre sus principios «*Every name within the scope of the Code (except for the names of «collective groups» and of taxa above the family group) is permanently attached to a name-bearing type»* (http://www.nhm.ac.uk/hosted-sites/iczn/code/). El International Code of Botanical Nomenclature que ha estado vigente hasta el año 2012 se basa en lo mismo y su principio II dice: «*The application of names of taxonomic groups is determined by means of nomenclatural types»* (*http://ibot.sav.sk/icbn/main.htm*). En el XVIII International Botanical Congress celebrado en Melbourne en 2011 se aprobó el *International Code of Nomenclature for algae, fungi, and plants* (http://www.iapt-taxon.org/nomen/main.php). Entre las principales novedades de este nuevo código, recientemente publicado, está el cambio de nombre, que ya no se llama botánico para no dar la impresión de que trata únicamente de plantas vasculares.

Ahora incluye los nombre de algas, hongos y plantas, escrito en minúsculas, pues no los acepta formalmente como grupos taxonómicos, sino como aquellos grupos que son tratados por ficólogos, micólogos y botánicos. Este código incluye también a los protistas fotosintéticos y a aquellos que, aunque no sean fotosintéticos, están taxonómicamente relacionados con ellos. Éste es caso de los dinoflagelados que comprende tanto especies autótrofas como heterótrofas. Otras novedades del nuevo código que afectan a la taxonomía de los dinoflagelados es que ya no hay obligación de publicar una diagnosis en latín sino que ésta puede estar escrita en latín o en inglés, y que no es indispensable publicar las descripciones en papel impreso para que éstas sean válidas, sino que también se pueden publicar en revistas en internet en formato pdf y que tengan ISSN o ISBN (Knapp *et al.*, 2011, McNeill *et al.*, 2011). Estas pequeñas novedades, abandonar el latín y aceptar internet, quieren darle un aire más moderno al código, pero éste mantiene el mismo principio muy conservador que caracteriza estos códigos en su artículo 7.2. «*A nomenclatural type (typus) is that element to which the name of a taxon is permanently attached, whether as the correct name or as a synonym. The nomenclatural type is not necessarily the most typical or representative element of a taxon*». El gran problema que representa el sistema basado en tipos es que, como el mismo código dice, el tipo nomenclatural no es necesariamente el representante más típico o representativo de un taxón. Este viejo enfoque tipológico con el que los taxónomos aún describen las especies, aun considerando éstas como linajes genealógicos cohesivos, es la causa de la frustración de muchos biólogos no taxónomos. Sin embargo, aunque la nomenclatura de las especies debe ser tipológica según el código, la descripción de la especie no debe ser tipológica (Dayrat, 2005) teniendo en cuenta la noción de linaje, que es un componente común de todos los conceptos de especie propuestos en los últimos 50 años (de Queiroz, 1998, de Queiroz, 2005).

Concepto de especie

Mientras que, como se ve, los códigos de nomenclatura son muy conservadores y de evolución muy lenta, las discusiones sobre conceptos de especie son muy activas y parecen no tener fin. Reflejando la polémica posterior a Darwin a finales del siglo XIX, Bessey (1908) trata este tema con gran pasión desde un extremismo nominalista al decir cosas como: «*Nature produces individuals, and nothing more*»; «*So species have no actual existence in nature. They are mental concepts and nothing more*»; «*Species have been invented in order that we may refer to great numbers of individuals collectively, instead of singly; therefore the number of species must be far less than the number of individuals*». Este debate se hizo más intenso a partir de la publicación de *Genetics and the Origin of Species* en 1937 por Dobzhansky en el que se introduce el concepto de barrera reproductiva. Su trabajo fue complementado por Ernst Mayr en (1942) en *Systematics and the Origin of Species* y otros posteriores donde establece y desarrolla el concepto biológico de especie

definido como «*species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from such other groups*» (Mayr, 1942). Esta definición fue posteriormente modificada ligeramente por el mismo Mayr in varias ocasiones eliminando «*actual or potentially*» y añadiendo «*a specific niche in nature*». Este concepto se basa firmemente en la ampliamente aceptada noción de especiación alopátrica. En el desarrollo de la discusión fundamentalmente académica sobre el concepto de especie se han definido multitud de conceptos de tal forma que la pregunta de qué es una especie ha llegado a ser un enigma filosófico. Fenchel y Finlay (2006) discutiendo el concepto de especie para microbios eucariotas (protistas) incluso parten de la idea de que no existe ningún concepto de especie con base teórica y que la importancia de nombrar especies radica en que así se organiza la información biológica, en especial la relativa a las propiedades funcionales y fenotípicas de los organismos. Se muestran pues, tan extremadamente nominalistas como Bessey (1908). La bibliografía sobre el tema es inmensa y continúa creciendo con gran disparidad de opiniones de tal forma que Mann (1999) se pregunta si no estaremos tratando de definir lo indefinible. En este caso, Mann se muestra claramente realista o esencialista cuando dice: «*... species are real -they do exist, at least among eukaryotes- and hence can be discovered. And exist because of the uncontroversial existence of sexual reproduction and meiosis*» (Mann, 1999). Las discusiones son a menudo muy apasionadas, como las críticas que Sokal & Corvello (1970) le hicieron al concepto biológico de especie tal como fue definido por Mayr (1963). Más recientemente, durante la celebración de la 12th International Conference on Harmful Algae en 2006 en Copenhague, David Mann y Tom Fenchel se enzarzaron en una muy agria discusión entre esencialismo y nominalismo.

Mann (1999) diferencia entre concepto de especie y la definición del mismo. Llega a la conclusión de que la mayoría de las discusiones no son sobre diferentes conceptos de especie en los que las diferencias entre autores, son pequeñas, sino sobre las diferentes definiciones del mismo concepto.

Tratando de ordenar esta discusión, Mayden (1997) revisa 22 conceptos de especie en uso en ese momento, algunos de ellos incompatibles en sus apreciaciones de la diversidad biológica, y llega a la conclusión de que únicamente hay un concepto teórico apropiado para las especies, el concepto evolutivo de especie. Para este autor, el resto de los conceptos de especie son secundarios y forman una jerarquía de directrices subordinadas al primer concepto y son esenciales para el estudio de las especies en la práctica. Los conceptos secundarios deberían usarse como herramientas operacionales para, a través de la variación en la diversidad natural, descubrir entidades de acuerdo con el concepto principal.

De Queiroz (1998) considera que para abordar el proceso de especiación hay que tener claro previamente qué es una especie. Hace también una revisión de muchos

conceptos de especie y llega a la conclusión, al igual que Mann (1999), de que aunque haya muchas definiciones de conceptos de especie no hay tantos conceptos diferentes como parece. Todos esos conceptos de especie, aunque tienen algunas pequeñas diferencias conceptuales, tienen algo en común: De un modo explícito o implícito todas consideran a las especies como segmentos de linajes evolutivos de poblaciones (de Queiroz, 1998).

Coyne & Orr (2004) en su libro sobre especiación discuten 9 conceptos de especie, de entre los muchos revisados por Mayden (1997) y De Queiroz (1998), y los agrupan en cuatro tipos, aunque estos autores se basen fundamentalmente en el concepto biológico de especie. (Tabla 1).

Para Steidinger (2010), todos los conceptos de especie se basan en poblaciones, excepto el morfológico y el fenético (taxonomía numérica) que se basan en individuos. Todos los conceptos o modelos incluyen una serie de caracteres descriptivos o identificadores que pueden ser reconocidos consistentemente aunque estos caracteres conservadores puedan variar algo en el tiempo y el espacio.

Toda esta multitud de definiciones de conceptos de especie no significa que todos sean incompatibles entre ellos, sino que representan un esfuerzo en alcanzar un concepto que sea universal y útil para todos los que lo usen. En muchos casos, unos derivan de otros tratando de resolver los problemas que dejan abiertos los anteriores. El mismo Mayr, a lo largo de su dilatada carrera fue modificando su definición original del concepto biológico de especie. Aparte de las discusiones conceptuales entre nominalistas y esencialistas, hay un acuerdo de utilizar las especies para clasificar los individuos dando lugar a la taxonomía. El mismo Bessey decía: «*She* (la naturaleza) *produces them in such a countless numbers that we are compelled to sort them into kinds in order that we may be able to carry them in our minds. This sorting is classification-taxonomy.*» (Bessey, 1908). Con el paso del tiempo, parece que esto es algo que aparentemente no se puede alcanzar, por lo que se está apostando por soluciones de compromiso, lo que se ha dado en llamar la taxonomía integradora (Will *et al.*, 2005, Dayrat, 2005) que se tratará más adelante.

CONCEPTOS DE ESPECIE UTILIZADOS EN LOS ESTUDIOS DE DINOFLAGELADOS.

El problema de definir especies, o cómo afrontarlo de un modo práctico, es muy diferente según con qué grupo de organismos se trate. No puede ser lo mismo para plantas, animales o bacterias. La mayoría de las discusiones teóricas son por parte de botánicos o zoólogos. Mayr definió el concepto biológico de especie basado en su experiencia como zoólogo. Al basarse e la reproducción sexual, quedan fuera de su aplicación aquellos organismos que no la tienen, como los procariotas, y en la práctica no se puede aplicar a

Tabla 1. Concepto biológico de especie y algunas alternativas propuestas (Coyne & Orr, 2004)

Base del concepto	Concepto	Definición
1. Cruzamiento	Concepto biológico de especie (BSC)	Las especies son grupos de poblaciones que se cruzan de modo natural y que están aisladas reproductivamente de otros grupos (Mayr, 1995).
2. Cohesión genética o fenotípica	Concepto de especie como grupo genotípico (GCSC)	Una especie es un grupo de distinguible individuos que tiene pocos o ningún elemento intermedio cuando está en contacto con otros grupos (Mallet, 1995).
	Concepto de especie de reconocimiento (RSC)	Una especie es la población más inclusiva de organismos de origen biparental que comparten un sistema de fertilización común. (Paterson, 1985).
	Concepto cohesivo de especie (CSC)	Una especie es la población más inclusiva de individuos que tienen un potencial de cohesión fenotípica a través de mecanismos intrínsecos de cohesión (Templeton 1989)
3 Cohesión evolutiva	Concepto ecológico de especie (EcSC)	Una especies es un linaje (o grupo de linajes muy próximos) que ocupa una zona adaptativa mínimamente diferente de la de otro linaje en su rango y que evoluciona separadamente de todos los linajes fuera de su rango (Van Valen, 1976).
	Concepto evolutivo de especie (EvSC)	Una especie es un linaje simple que de poblaciones o individuos que descienden de un ancestro común, que mantienen su identidad frente a otros linajes y que tiene sus propias tendencias evolutivas y destino histórico (Wiley, 1978. modificado de Simpson, 1961)
4. Historia evolutiva	Concepto filogenético de especie 1 (PSC1)	Una especie filogenética es un cluster basal de organismos que son diagnosticablemente distintos de otros clústeres y dentro del cual hay una relación de ancestros (Cracraft, 1989).
	Concepto filogenético de especie 2 (PSC2)	Especie es el grupo filogenético más pequeño que tenga un ancestro común (de Queiroz & Donoghue, 1988)
	Concepto filogenético de especie 3 (PSC3) o Cocepto genealógico de especie	Una especie es un grupo basal y exclusivo de organismos cuyos genes se relacionan entre ellos más que con los de aquellos fuera del grupo, y que non contienen ningún grupo exclusivo dentro de él (Baum and Donoghue, 1995; Shaw 1998)

aquellos que, aunque se suponga que sí la tienen, no se le conoce. Este problema está resuelto en otros conceptos como el evolutivo o el filogenético. Burger (1975) discute las dificultades que encontró para aplicar el concepto biológico de especie a las multiples especies de robles basadas en el concepto clásico de especie morfológico o fenético. Entre los robles son muy frecuentes los híbridos entre especies que son fértiles, por lo que según el concepto biológico de especie serían una sola especie al poder tener intercambio genético. Este caso fue más tarde tratado por Van Valen (1976) para definir el concepto ecológico de especie tratando de resolver los problemas descritos por Burger.

La discusión con los protistas discurre un poco aparte de los otros grupos al tratarse de casos muy diferentes de los que se pueden encontrar con animales o plantas. Dentro de lo que consideramos protistas la diversidad de grupos es mucho mayor que la que se encuentran los botánicos o zoólogos por lo que también aquí nos encontramos con disparidad de criterios según los grupos con que se trabaje.

Mann (1999) discute el concepto de especie en diatomeas, y siendo él como es, esencialista, asume el concepto biológico de especie según el cual, dentro de una especie hay flujo genético y recombinación, pero cuando no hay reproducción sexual, o ésta no se conoce, la variación es jerárquica, de tal forma que el rango de un grupo al que se considera «especie» es algo convencional en base a su utilidad. Por esta razón la sistemática de los fósiles, entre los que las diatomeas son un grupo muy estudiado, será siempre deficiente al no poder estudiar el flujo genético. Mann y Evans (2008) consideran que la meiosis y la reproducción sexual son probablemente caracteres simplesiomórficos para todos los protistas por lo que, en teoría, para todos se podría utilizar el concepto biológico de especie, algo que, por ejemplo, no es posible con cianobacterias. Sin embargo, actualmente estamos muy lejos de conocer el ciclo sexual de la inmensa mayoría de las especies de protistas, por lo que aún los esencialistas tienen que utilizar criterios de los nominalistas a la hora de definir y describir una especie.

De entre todos los conceptos de especie definidos, se discuten a continuación, en más detalle, aquellos que más se han usado para estudios taxonómicos de dinoflagelados.

Concepto morfológico de especie

Este ha sido hasta ahora el concepto de especie más utilizado en dinoflagelados. Como para seguir desarrollando la taxonomía no se podía esperar al fin de la discusión sobre qué es una especie, y cuando las técnicas de observación de los protistas eran muy primitivas, desde el punto de vista práctico, en los estudios taxonómicos de los dinoflagelados, se ha utilizado el concepto morfológico de especie ya que, si bien el concepto biológico de especie de Mayr es fácilmente utilizable en animales, -él era un

zoólogo- no es aplicable en la mayoría de los protistas ya que, o carecen de reproducción sexual o simplemente no se le conoce y únicamente se conoce la asexual.

Mayden (1997) recoge varias definiciones del concepto morfológico de especie:

«Las especies son los grupos más pequeños que son consistente y persistentemente distintos y distinguibles por medios ordinarios». (Cronquist, 1978: 15, citado en Mayden (1997)).

«Las especies pueden ser definidas como los tipos de organismos reconocidos fácilmente, y en el caso de las plantas y los animales macroscópicos su reconocimiento debe basarse en la observación simple, como la que cualquier persona inteligente pueda hacer con la ayuda de, por ejemplo, solamente de una buena lupa de mano». (Shull, 1923: 221, citado en Mayden (1997)).

«Las poblaciones naturales más pequeñas permanentemente separadas unos de otras por una discontinuidad clara en la serie de biotipos». (Du Rietz, 1930: 357, citado en Mayden (1997)).

«A species is a community, or a number of related communities, whose distinctive morphological characters are, in the opinion of a competent systematist, sufficiently definite to entitle it, or them, to a specific name». (Regan, 1926: 75, citado en Mayden (1997)). [Nota: aquí a la palabra «comunidad» no se le da el significado que actualmente tiene en ecología, sino que se refiere simplemente a un conjunto de individuos]

Las especies son los grupos más pequeños que pueden ser definidos repetidamente por características que son relativamente fáciles de distinguir (Graham & Wilcox, 2000).

La decisión, entonces, de considerar si las diferencias morfológicas encontradas entre diversos individuos son suficientes o no para considerarlas como definitorias de especies diferentes compete a los «taxónomos reconocidos». Esta subjetividad hace que haya diferencias notables entre taxónomos a la hora de agrupar individuos en una o más especies. Aquellos que son partidarios de grandes unidades son denominados en inglés «*lumpers*» (El Cambridge English Dictionary, define *lump* como «*consider or deal with as a group*». Por ejemplo: «*Children of various abilities are lumped together in one class»* por lo que podrían denominarse en castellano como «agrupadores») y los que, por el contrario, prefieren las definiciones precisas, y crear nuevos taxones para clasificar los individuos, son calificados como «*splitters*» («separadores»). Tal vez haya sido Darwin, en una carta a J.D. Hooker en 1857, el primero en utilizar estos términos. En el marco de un trabajo sobre el tratamiento que le daban a las especies y variedades en diversas floras en el mundo, le escribe a Hooker:

«*I am got extremely interested in tabulating, according to mere size of genera, the species having any varieties marked by Greek letters or otherwise: the result (as far as I have yet gone) seems to me one of the most important arguments I have yet met with, that varieties are only small species-or species only strongly marked varieties. The subject is in many ways so very important for me; I wish much you would think of any well-worked Floras with from 1000-2000 species, with the varieties marked. It is good to have hair-splitters and lumpers. (Those who make many species are the «splitters,» and those who make few are the «lumpers.»).* (Cartas de Darwin: http://www.turtlereader.com/authors/charles-darwin/the-life-and-letters-of-charles-darwin-day-153-of-188/).

Tal vez el más reconocido taxónomo reciente de dinoflagelados tecados fue Enrique Balech. Él se consideraba a sí mismo como un «*splitter*» y decía así en una carta que me dirigió en 1993 en el marco de una discusión sobre la sinonimia entre los dinoflagelados *Alexandrium minutum* y *Alexandrium lusitanicum*:

«El problema de qué es una especie de protistas es verdaderamente difícil de dilucidar. Sus dudas se parecen bastante a las de Anderson aunque con base distinta. Él presentó para publicación, con otros, un trabajo donde hay una discusión de la especie de Alexandrium*. Él prefiere una especie mucho más amplia de lo que yo mantengo, basado en caracteres de morfología tecal. Y mis pretendidas especies serían mantenidas como variedades. Creo que los dos criterios son aceptables mientras no tengamos una regla para definir la especie. En este caso soy un «splitter» porque, siguiendo el criterio de Kofoid y para evitar largos nombres binomiales, prefiero llamar especie a todas las entidades taxonómicas que pueden diferenciarse por aunque sea un pequeño carácter pero que se mantiene constante y se transmite genéticamente. Hay dos razones para esta decisión: primero el abuso que han hecho muchos botánicos de estas categorías infraespecíficas llegando a taxones con 5 palabras (algunos no se conforman con la «forma» sino que quieren agregar un nombre para la «subforma»!. La segunda razón es que mientras no tengamos más conocimientos sobre el género y criterios taxinómicos más sólidos conviene manejar criterios taxinómicos estrechos. Si hoy mantenemos nombres diferentes para dos poblaciones que tienen caracteres diferenciales, por pequeños que ellos sean, y si mañana se nota la identidad específica de ambos, la reunión de datos no presenta dificultades. Si, por el contrario, tomamos las dos, digámosles sps. A.b. y A.c. y, desde ya presentamos todos los estudios referidos a estas «formas» bajo un mismo nombre y más tarde encontramos que hay diferencias ecológicas o de otro orden, no hay manera de discriminar en todo el material que se estudió bajo el mismo nombre. Las variaciones de toxinas (por ej. porcentajes de las distintas PSP) parecen demasiado variables. Pero eso no quita su alto interés. Lo malo es que, como dijo el protozoólogo norteamericano Corliss, hoy nos manejamos con varias sistemáticas: basadas en*

pigmentos, en estructuras nucleares, en infraciliaturas, en estructuras flagelares y ciliares, en secuencias ribosomales y, agrego, en toxinas. Todas me parecen válidas (olvidaba la basada en quistes). Pero, por ahora, la única de utilización universal, en todas las condiciones (por ej. material fijado y de campañas oceanográficas, colecciones (muestras) antiguas, sedimentos, es la morfología tecal. Y, desde luego, es la única referencia bibliográfica que tenemos de las décadas anteriores.»

Un ejemplo claro de este tipo de problemas apuntados por Balech, causados por los «*lumpers*», y que se han derivado de agrupar especies diferentes en una, es el de la aparente sinonimia entre *Prorocentrum mexicanum* y *Prorocentrum rathymum*. *P. mexicanum* fue descrito por un exiliado republicano gallego tras la guerra civil española, Bibiano Fernández Osorio-Tafall (1942) en una revista mejicana en español, por lo que fue ignorado durante muchos años por el mundo científico anglosajón. Steidinger (1983) encontró esta descripción y consideró que *P. rathymum*, descrito por Loeblich *et al* (1979) con posteridad a *P. mexicanum*, era un sinónimo de este último. De esta forma, a partir de esa fecha, cuando alguien encontraba *P. ratymum* lo citaba como *P. mexicanum* hasta que, después de dos décadas, se demostró que sí se trataba de dos especies diferentes (Cortés-Altamirano & Sierra-Beltrán, 2003) y que la supuesta sinonimia era un error. Es difícil ahora discernir de cual de las dos especies se trataba cuando durante los años que se consideraron sinónimos se citó *P. mexicanum,* perdiéndose de esta manera mucha información.

Otro ejemplo es el de la pareja *Alexandrium minutum* y *Alexandrium lusitanicum* al que aludía Enrique Balech en su carta. Balech (1985) describió *A. lusitanicum* como una especie nueva pues la descripción original de *A. minutum* (Halim, 1960) no le daba la suficiente información y él encontró en su especie algunos detalles morfológicos que, en base a su experiencia, consideró que podían ser significativos. Una vez probada la sinonimia entre ambas especies (Franco *et al.*, 1995) toda la información que había publicada sobre *A. lusitanicum*, principalmente de autores portugueses e italianos, se puede considerar como de *A. minutum* por lo que no se perdió información, dándole la razón a Balech sobre las ventajas prácticas derivadas de aplicar los criterios de los «splitters» sobre los de los «lumpers».

Esta es una prueba clara de que la descripción de especies en base a su morfología es fuertemente subjetiva, de tal forma que lo que algunos autores consideran como «especies buenas» («good species»), otros las consideran como sinónimos. O como decía Darwin en The Origin of Species *«Hence, in determining whether a form should be ranked as a species or a variety, the opinion of naturalists having sound judgment and wide experience seems the only guide to follow»*(Darwin, 1859) (pág. 65), opinión, que como se dijo más arriba, mantenía Regan (1926).

INTRODUCCIÓN

En protistas, la diferenciación de especies utilizando el concepto morfológico de especie, está fuertemente ligada a la técnica de observación utilizada. Por ejemplo, en especies de diatomeas del género *Pseudo-nitzschia*, utilizando microscopía de luz con muestras vivas en agua, difícilmente se pueden distinguir especies y solamente se pueden hacer grupos en base a sus dimensiones. Por ejemplo, las de más de 3 µm de ancho valvar, son del grupo «seriata» y las de menos de 3 µm, del grupo «delicatíssima», dos categorías sin entidad taxonómica. Si se limpian los frústulos con ácido y se incluyen en resina, con el microscopio de luz y con contraste de fases o con iluminación Normarsky, ya se pueden identificar algunas especies en base a si tienen o no, interespacio central y por el número de fíbulas y estrías en 10 µm. Sin embargo, hay especies en las que con microscopía de luz no se pueden resolver las estrías, pues no tiene suficiente resolución, para lo que es necesario el uso de microscopía electrónica de barrido (SEM). La microscopía electrónica de transmisión (TEM) permite ver los detalles de la estructura de los poros y diferenciar especies que no es posible con las técnicas anteriores. Por ejemplo, donde antes se consideraba que había una sola especie, la *Pseudo-nitzschia pseudodelicatissima*, surgieron dos más, la *P. caliantha* y la *P. caciantha* (Lundholm & Moestrup, 2003). Aún así, todavía quedan especies que únicamente se diferencian genéticamente. *P. delicatissima* se consideraba como una morfoespecie bien definida, pero el análisis genético ribosomal dió lugar al descubrimiento de una diversidad genética no esperada que sugiere la existencia de grupos aislados reproductivamente (Orsini *et al.*, 2004), es decir, diferentes especies si se utiliza el concepto biológico de especie y no el morfológico. Amato *et al*. (2007) estudian diversas especies del género *Pseudo-nitzschia* del grupo delicatissima y mediante microscopía electrónica distinguen ocho posibles especies, mientras que por microscopía de luz únicamente pueden llegar a hacer dos grupos de especies (Fig. 2). Con esto queda claro que los resultados de aplicar el concepto morfológico de especie en protistas es totalmente dependiente de las técnicas de observación utilizadas.

En concreto, entre los dinoflagelados, el género *Alexandrium* tiene especies que en su morfología gruesa son exactamente iguales si se observan con microscopio de luz con campo claro. Sin embargo, si se diseccionan sus tecas y se obsevan con contraste de fases, o bien se utiliza una tinción fluorescente de las placas, es posible distinguir especies. De esta forma, hasta hace no muchos años, cualquier *Alexandrium* aparecía citado como *Gonyaulax tamarensis* (hoy *Alexandrium tamarense*) cuando hoy hay descritas varias decenas de especies de este género (Balech, 1995) pero que necesitan de un buen equipo de microscopía y experiencia para poder ser identificadas. Lo mismo ocurre con el género *Gambierdiscus* en el contínuamente se están describiendo nuevas especies, y en el que incluso se ha llegado a la conclusión de que en la descripción original de la especie que definió al género, *G. toxicus* había varias especies mezcladas y fue necesario redescribirlo (Litaker *et al*., 2009).

Una dificultad añadida al concepto morfológico de especie es la existencia de fases del ciclo vital muy diferentes. Los quistes de dinoflagelados fueron considerados durante mucho tiempo por los paleontólogos como un grupo biológico extinto denominado como «Hystricosphaeras» hasta que David Wall y Barrie Dale publicaron en Nature su famoso artículo *«Living Fossils» in Western Atlantic Plankton,* (Wall & Dale, 1966) en el que describen la eclosión de quistes aislados de sedimento reciente que daban lugar a fases móviles de dinoflagelados muy conocidos. Otros ejemplos de diferentes fases del ciclo vital consideradas como diferentes especies, se encuentran en especies de dinoflagelados del género *Dinophysis*. En algunos pares de especies tales como *D. acuta*/*D. dens*, *D. caudata*/*D. diegensis* o *D. acuminata*/*D. skagii*, se observó que simplemente eran diferentes fases del ciclo vital de la misma especie en las que las células tenían diferentes tamaños y silueta tecal (Reguera & González-Gil, 2001, MacKenzie, 1992, Rodríguez *et al.*, 2012).

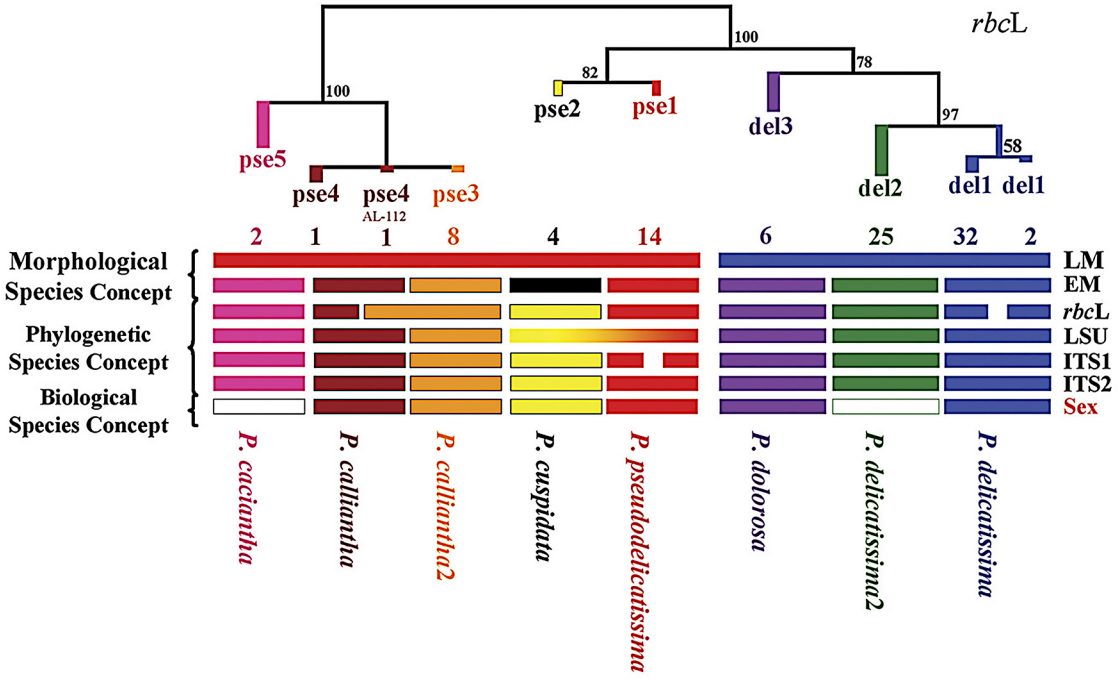

Figura 2. Árbol inferido de las secuencias rbcL. Los números en la primera fila representan el número de cepas analizadas. Las barras horizontales indican los patrones de agrupamiento reconocidos por morfología al microscopio de luz y ultrastructural por microscopía electrónica de transmisión (TEM), marcadores moleculares (que aparecen en el lado derecho), y la compatibilidad sexual. El rectángulo negro lleno indica que los análisis ultrastructurales no se llevaron a cabo, el rectángulo vacío verde que se llevaron a cabo experimentos de apareamiento, pero nunca produjeron fases sexuales y el rectángulo negro vacío que no se llevaron a cabo experimentos de apareamiento (Amato *et al* 2007).

Elbrächter (2003) cita una frase de Von Stosch cuando, en una ocasión, estaban discutiendo sobre el complejo de especies *Dissodinium- Pyrocystis: «You only know a species if you know its complete life cycle.»* Si se sigue este criterio, solamente conocemos el 1% del total de taxa recientes de dinoflagelados, si es que realmente se conoce alguno. La mayoría de los dinoflagelados se han descrito a partir de muestras fijadas, años después de que éstas hayan sido tomadas, y por lo tanto se desconocen algunas características biológicos importantes tales como la presencia o ausencia de cloroplastos, la forma del núcleo, el modo de división celular, o el ciclo de la vida. Únicamente conocemos su forma y, si son tecados, solamente las principales placas de su teca, ya que de muchas especies de estos dinoflagelados no conocemos ni sus placas cingulares ni las sulcales (Elbrächter, 2003).

Concepto biológico de especie

Como ya se dijo más arriba, este concepto fue formalmente definido por Mayr (1963) aunque posteriormente lo fue refinando. El concepto biológico de especie es aquel según el cual las especies se identifican como grupos de poblaciones que se cruzan entre ellas y están aisladas reproductivamente de otras, y por lo tanto representan unidades evolutivas independientes.

Para poder utilizarlo es necesario que exista, y se conozca, la reproducción sexual de las especies. Como de la mayoría de las especies de dinoflagelados no se conoce su ciclo sexual, no es posible aplicar a todos los dinoflagelados el concepto biológico de especie. Incluso en aquellos casos en los que sí se conoce su ciclo sexual puede resultar complicada la utilización del concepto biológico de especie al ser difícil determinar donde se encuentra la barrera reproductiva entre dos especies cercanas. Aunque este concepto de especie es el más reconocido entre los biólogos, no se utiliza para describir especies de dinoflagelados debido a las dificultades prácticas de aplicarlo, por lo que se buscan alternativas factibles pero siempre con este concepto en el fondo, considerando que lo ideal sería poder probar que las especies descritas con criterios morfológicos o filogenéticos cumplen también con el concepto biológico de especie. Sin embargo para probar las fronteras entre especies cuando no es posible hacerlo directamente, existen métodos para estimar indirectamente el flujo genético dentro y entre hipotéticas especies (Sites & Marshall, 2004).

El aislamiento reproductivo entre dos especies depende de las barreras reproductivas que haya entre ellas. Estas barreras pueden estar a diferentes niveles dependiendo de la proximidad evolutiva entre las especies. Dentro del esquema clásico de ciclo vital de los dinoflagelados que forman quistes sexuales, la primera barrera sería aquella que no permite que dos gametos se fusionen. La siguiente barrera sería aquella en la que si hay fusión de gametos, el zigoto resultante no forma quiste. Otra barrera sería en la eclosión del quiste.

Si eclosiona, la célula resusltante no dará lugar a una descendencia, y en el caso de que sí la diese, se trataría de la misma especie. Brosnahan *et al.* (2010) intentaron cruces entre diferentes grupos del complejo de especies *Alexandrium tamarense / catenella* y obtuvieron un cruce entre una cepa tóxica del grupo I y otra no tóxica del grupo III. Aunque formaron quiste y éste eclosionó, no se observaron más de tres divisiones probando que según el concepto biológico de especie los grupos I y III son dos especies diferentes.

Concepto filogenético de especie

Este concepto tiene varias definiciones. Cracraft, (1989) (págs. 34-35) define una especie filogenética como: «*Phylogenetic species is an irreducible (basal) cluster of organisms, diagnosable distinct from another such clusters, and within which there is a parental pattern of ancestry and descent*». El problema para aplicar este concepto de especie radica en el modo de reconocer dos clústeres como distintos (Fig. 3).

Con la expansión de las técnicas moleculares de secuenciación de ácidos nucleicos en los años 90, se abrieron nuevas perspectivas para la diferenciación de especies pero las nuevas herramientas no aportaron nada nuevo para solucionar el problema conceptual. Desde muy temprano, las nuevas técnicas se utilizaron más para estudiar la filogenia que para diferenciar especies (Sogin *et al.*, 1986) y éste sigue siendo el mayor interés de los trabajos que utilizan secuenciaciones genéticas de protistas. Se trata de hacer el árbol publicado por Darwin en su *«Origin»* pero en base a las secuencias obtenidas en los laboratorios mediante el uso de herramientas estadísticas y bioinformáticas. La gran cantidad de secuencias genéticas actualmente disponible y fácilmente accesible a través del GenBank (http://www.ncbi.nlm.nih.gov/), está permitiendo, y obligando, una revisión de la taxonomía sistemática de los dinoflagelados que anteriormente se había basado únicamente en morfología con algunas aportaciones bioquímicas. Sournia (1986) ya apunta a que la mayoría de las especies de dinoflagelados están aún por describir y Litaker (2007) destaca que la frecuencia y diversidad de secuencias de DNA de dinoflagelados sin clasificar sugiere la existencia de numerosas especies todavía por evaluar morfológicamente. El número de especies que puede haber en el planeta es motivo de gran discusión y se estima que el número de especies de eucariotas, excluyendo bacterias, puede estar en un rango tan amplio como de 2 a 100 millones (Costello et al., 2013)

Después de siglos de trabajo taxonómico, los sistemáticos han intensificado recientemente los esfuerzos para convertir a la taxonomía desde una ciencia no cuantitativa a una numérica y analítica (Lim *et al.*, 2012). Para delimitar las especies, muchos de los nuevos métodos diseñados para las secuencias de DNA buscan detectar un cambio en la cualidad de la señal evolutiva que pueda reflejar la distinción entre la variación intraespecífica y la separación interespecífica (Lim et al., 2012, Coleman, 2009)

Litaker *et al.* (2007), conscientes de que, para identificar posibles especies, actualmente no existen criterios sistemáticos para utilizar taxonómicamente datos de secuencias no asignadas a una especie determinada, y que a su vez puedan servir de base para plantear hipótesis relativas a la taxonomía, diversidad, distribución y toxicidad de los dinoflagelados, analizaron las secuencias de 81 especies de 14 géneros de dinoflagelados para evaluar si, para desarrollar criterios para reconocer posibles especies antes de su evaluación y clasificación morfológicas, se pueden utilizar las distancias

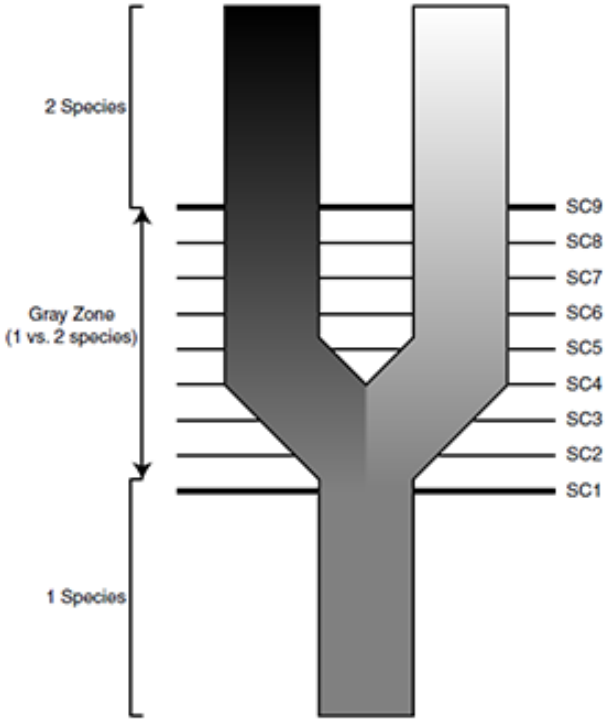

Fig. 3. Representación muy simplificada del proceso de divergencia de un linaje (especiación) que ilustra los conflictos ocasionados por la adopción, como propiedades necesarias de las especies, de diferentes propiedades contingentes a linajes metapoblacionales. El oscurecimiento progresivo y aclarado de los linajes hijos representan su divergencia progresiva en el tiempo (de abajo hacia arriba), y las líneas numeradas etiquetadas SC representan los momentos en que los linajes hijos adquieren propiedades diferentes entre sí (por ejemplo, cuando se convierten en fenotípicamente distinguibles, diagnosticables por una diferencia de un carácter fijo, mutuamente monofiléticos, incompatibles reproductivamente, ecológicamente distintos, etc).
Antes de la evolución de la primera propiedad (SC1), los autores están de acuerdo en que hay una sola especie, y después de la evolución de la última propiedad (SC8), ellos estarán de acuerdo en que hay dos. Entre estos eventos, sin embargo, habrá desacuerdo entre los autores acerca de si se trata de una o dos especies. Los desacuerdos son el resultado de la adopción por los autores de diferentes criterios como base para sus definiciones de especie. (de Queiroz, 1998, de Queiroz, 2005).

genéticas simples y no corregidas (*p*) utilizando secuencias ITS1/5.8S/ITS2 (región ITS) del DNA ribosomal. Estos autores llegan a la conclusión de que, para esas especies de dinoflagelados estudiadas, las distancias genéticas intraespecíficas entre copias de la región ITS (valores de *p* menores 0,021 substituciones por sitio) fueron consistentemente menores que las observadas entre especies (p = 0,042 - 0,580). Sus resultados indican que para delinear la mayoría de las especies de dinoflagelados podría utilizarse una distancia genética interespecífica, sin corregir, de p = 0,04. Sin embargo también consideran, que las especies evolucionadas recientemente, puedan tener valores de *p* menores de 0,04 y que, entonces requerirían análisis morfológicos y genéticos más detallados para resolverlas. Nishimura *et al*., (2013), utilizando secuencias de la SSU rDNA, las regiones D8–D10 de la LSU rDNA y la región ITS, encuentran cinco filotipos/especies de *Gambierdiscus* en aguas japonesas. De ellos, únicamente una se puede asignar a una especie ya descrita, el *G. australes*, mientras que otras tres serían nuevas especies por describir en base a las valores de *p* encontrados al comparar sus secuencias con las previamente publicadas. En el quinto caso encuentran que la distancia *p* de sus secuencias es de 0,002 con *G. yasumotoi* y de 0,121 con *G. ruetzleri*, especies que Litaker et al. (2009) consideran diferentes en base que a que *p* entre ellas es de 0,004. Aunque Nishimura *et al.,* (2013) no proponen formalmente que *G. ruetzleri* sea un sinónimo de *G. yasumotoi* y optan simplemente por llamarle a sus cepas *G*. cf. *yasumotoi,* si la diferencia con ambas especies es menor que 0,004 éstas serán a su vez sinónimos. Las diferencias genéticas encontradas entre los filotipos de las especies lenticulares de *Gambierdiscus* han permitido encontrar sutiles diferencias morfológicas entre ellos dando lugar a la descripción de *Gambierdiscus scabrosus* Nishimura, Sato y Adachi (Nishimura *et al.*, 2014).

Esta misma línea de investigación es la que desarrolla el laboratorio de Jang-Seu Ki en Corea a la hora de delimitar especies de dinoflagelados del género *Peridinium* (Ki *et al.*, 2011) o diatomeas de los géneros *Cyclotella* and *Discostella* (Jung *et al.*, 2010). Los datos de filogenia molecular también inducen a cambios en la la adscripción de especies a géneros como, por ejemplo, la transferencia de *Cochlodinium geminatum* al género *Polykrikos* (Qiu *et al.*, 2013).

Sin embargo hay que ser muy cautos a la hora de utilizar secuencias. Coleman (2005) estudiando el complejo de especies de *Paramecium aurelia sensu lato* llegó a la conclusión de que si dos organismos se cruzan y tienen una F1, sus secuencias de ITS2 son idénticas. Por el contrario, el que dos cepas tengan idénticas secuencias ITS2 no siempre implica que se puedan cruzar. Este es, por ejemplo, el caso de dos especies que morfológicamente se han adscrito a diferentes géneros: *Scrippsiella hangoei* y *Peridinium aciculiferum* (Logares *et al.*, 2007). La primera habita el Mar Báltico mientras que la segunda se encuentra en lagos templados del norte. Mediante microscopía electrónica se han observado diferencias interespecíficas significativas en la morfología externa de las células aunque

éstas tengan una tabulación similar. Experimentalmente se ha observado que *S. hangoei* puede crecer en un amplio rango de salinidades desde 0 a 30, mientras que *P. aciculiferum* sólo lo puede hacer entre 0 y 3. A pesar de estas diferencias fenotípicas y de segregación de ambientes, ambas especies tienen idénticas secuencias del DNA ribosomal (ITS1, ITS2, 5.8s, SSU y LSU parcial) (Logares *et al.*, 2007).

Hasta hace poco se consideraba que un cambio en una base de la ITS2 era independiente de las demás bases. Sin embargo al formarse una estructura secundaria del RNA en forma de hélices, un cambio en una base afecta a esa estructura de tal forma que si se tienen en cuenta las estructuras secundarias del RNA ribosomal facilitarán el alineamiento de las secuencias. Cuando hay una sustitución de una base que se ve compensada con la correspondiente base con la que estaba emparejada en la hélice se dice que hay un cambio de base compensatorio (CBC = *Compensatory Base Change*). Se ha observado empiricamente que si dos organismos tienen algún CBC en la hélice II o la hélice III de la estructura secundaria de la ITS2, éstos no se pueden cruzar. Un CBC ocurre cuando dos nucleótidos de una región pareada mutan de tal forma que el par se mantiene (por ejemplo C-G muta a A-U (Müller *et al.*, 2007). Sin embargo, si entre dos organismos no hay ningún CBC, esto no implica que necesariamente tengan que pertenecer a la misma especie. Este hecho que permite utilizar una extraordinaria herramienta para diferenciar especies según el concepto biológico de especie sin tener que hacer experimentos de cruces, ha sido sobrevalorado de tal forma que Wolf *et al* (2013) lleguen incluso a sugerir el concepto CBC de especie, sin reconocer que no altera el concepto biológico de especie en absoluto aunque sea una gran herramienta para su aplicación. Cada vez son más frecuentes los trabajos que utilizan los CBC para diferenciar especies de dinoflagelados tales como *Coolia malayensis* de *Coolia monotis* (Leaw *et al.*, 2010), o diatomeas como *Pseudo-nitzschia* spp. (Amato *et al.*, 2007, Lundholm *et al.*, 2012, Orive *et al.*, 2013).

Actualmente las estimaciones de diversidad se basan en el número de especies o en la asignación de unidades taxonómicas operacionales (OTU, Operational Taxonomic Unit). Por lo tanto, cualquier variación en este número no sólo afectará a las estimaciones de la diversidad, sino también a las hipótesis ecológicas que puedan derivarse de dichas observaciones (Gazis *et al.*, 2011). Hay diversas definiciones de OTUs. Para Sokal (1966), una OTU puede estar constituida por individuos como tales, individuos representando especies o rangos taxonómicos superiores tales como géneros o familias de plantas o animales o abstraciones estadísticas de grupos taxonómicos de rango superior. Para Diez *et al*. (2001), por ejemplo, clones que produzcan el mismo patrón RFLP (Fragmentos de DNA del mismo tamaño) se agrupan y se consideran miembros del la misma OTU. Para Edgar (2013) OTUs son grupos de secuencias que se pretende que correspondan a clados taxonómicos o grupos monofiléticos.

Con el fin de detectar posibles especies o OTUs utilizando el concepto filogenético de especie, Gazis *et al* (2011) combinaron datos moleculares con ecológicos y llegaron a la conclusión de que, al menos para hongos, la ITS sola, generalmente subestima el número de especies que se se detectarían utilizando otros loci nucleares. Estos resultados cuestionan el uso de las secuencias de ITS y umbrales arbitrarios de divergencia para la delimitación de especies como se hace normalmente para clasificar microbios dentro de la misma o diferente OTU (Wooley *et al.*, 2010, Sokal, 1966).

El concepto filogenético de especie tiene una gran atracción para estudios ecológicos (Caron, 2013, Caron *et al.*, 2004, Caron *et al.*, 2009, Díez et al., 2001) y no hay que confundirlo con el uso de la ecología para la delimitación de especies, como se trata en el siguiente apartado.

Concepto ecológico de especie

Fue definido formalmente por Van Valen (1976) trabajando con robles como: *«A species is a lineage (or a closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range.»*

Por linaje se entiende un clon o una secuencia de poblaciones de ancestros y sus descendientes, entendiendo por población a un grupo de individuos que entre ellos intercambian genes reproductivamente, y lo hacen con más frecuencia que con individuos de fuera de la población. Los linajes están relacionados estrechamente si han ocupado la misma zona adaptativa desde su último ancestro común . Si su zona de adaptación ha cambiado desde entonces, los linajes también estarán estrechamente relacionados, si las nuevas adaptaciones han sido transferidas entre los linajes en lugar de originarlas cada uno por separado. Una zona adaptativa es una parte del espacio de recursos junto con todo lo relacionado con depredación y parasitismo en el grupo considerado . Es parte del medio ambiente, distinto del taxón que pueda ocuparlo, y existe independientemente de cualquier habitante que pueda tener. La palabra «zona», aunque está arraigada, tal vez sea desafortunada ya que sugiere la existencia obligatoria de fronteras naturales o discontinuidades en el espacio de recursos. Los límites de una zona adaptativa se pueden fijar y se mantendrán cualqueira que sean las especies que la habiten.

Recientemente se han utilizado sistemas de información geográfica (GIS) para ayudarse de datos ecológicos en la delimitación de especies. Raxworthy et al. (2007) utilizando el concepto de nicho descrito en Hutchinson (1957) hacen un modelo utilizando variables climáticas como variables para definir diferentes nichos y de esta forma estudiaron las distribuciones de diferentes especies de geckos dirunos de Madagascar. Combinando los resultados de los modelos con datos moleculares y morfológicos, pudieron

elevar tres taxones considerados previamente como subespecies, al rango de especies y describir una nueva especie. De este modo llegaron a la conclusión de que el modelado de nichos ecológicos ofrece un gran potencial para la delimitación de especies, especialmente en aquellos grupos taxonómicos con baja capacidad de dispersión y endemismo localizado y también para grupos cuyas distribuciones son poco conocidas. En particular, los modelos de nichos ecológicos son especialmente sensibles para la detección de especiación parapátrica reciente que haya sido impulsada por divergencias ecológicas, cuando los gradientes ambientales de especiación están representados dentro de los modelos de nicho ecológico.

Rissler & Apodaca (2007) utilizando métodos similares también basados en modelos de nicho ecológico llegan a la conclusión de que algunas variedades de salamandra negra del California deberían ser elevadas a rango de especie en base a datos moleculares, morfológicos y ecológicos.

ESPECIES CRÍPTICAS, SEMICRÍPTICAS Y PSEUDO-CRÍPTICAS.

En el curso de la especiación, en aquellos casos de organismos en los que la morfología es importante, como por ejemplo aquellos en los que se usan señales visuales para interacciones sociales, selección de pareja o predación por animales provistos de visión, es de esperar que la correlación entre especiación y diferenciación morfológica sea importante (Coyne & Orr, 2004, Mann & Evans, 2008) mientras que en otros grupos de organismos en los que la morfología juegue un papel menos importante, habrá una relación menos consistente entre la morfología y los límites entre especies.

Utilizando el concepto morfológico de especie se observó que dentro de lo que se consideraba como una especie definida morfologicamente, a veces se encontraban diferencias considerables entre secuencias genéticas de sus indivíduos que permiten agruparlos como linajes moleculares si éstos son monofiléticos por loci no ligados entre sí. Frecuentemente se observa que estos linajes se corresponden con diferencias biogeográficas o fisiológicas suficientemente importantes como para considerarlos especies distintas.

Si realmente estas especies no se pueden distinguir morfológicamente con los métodos habitualmente utilizados se considera que éstas son especies crípticas.

La literatura sobre especies crípticas es muy abundante, y no siempre se entiende lo mismo por esta palabra. Aquí seguiremos las definiciones sugeridas por Mann y Evans (2008): Las especies son *crípticas* si no es posible identificar a los individuos de un modo consistente y preciso en base únicamente de la morfología.

Las especies se consideran como *semicrípticas* si sus individuos se pueden identificar de un modo consistente y preciso en base a su morfología, sólo si se conoce su procedencia, ya sea en cuanto a su origen geográfico o a las características de la población.

Las especies son *pseudocrípticas* si los individuos pueden ser identificados a partir de la morfología, siempre que se haga con el suficiente cuidado, pero son tan parecidos que hay una alta probabilidad de errar en su identificación, incluso aunque ésta sea hecha por un científico competente.

Frecuentemente ocurre que después de reconocer la existencia de especies crípticas, se encuentran pequeños detalles morfológicos a los que, en otras circunstancias, no se le concedería mayor importancia, pero que concuerdan con las especies que sugieren las secuencias genéticas. En ese caso pasarían a considerarse como pseudocrípticas. Medlin *et al.,* (1991), utilizando las, que en ese momento, eran nuevas técnicas, encontraron que dentro de lo que se consideraba la especie de diatomea *Skeletonema costatum*, había en realidad dos especies por lo que describieron una nueva, *Skeletonema pseudocostatum*, para distinguirla de *S. costatum* con la que anteriormente se confundía. Cuando tras ese trabajo se pueden diferenciar morfológicamente, aunque sea por un pequeño detalle, ya no se puede hablar de especies crípticas. En una revisión del concepto de especie en las diatomeas, Mann (1999) señaló que en realidad no se habían encontrado ningunas especies crípticas de verdad y que tan solo se trataba de especies que eran muy difíciles de distinguir a simple vista.

Hay otras formas de definir especies crípticas. Para Schönrogge *et al.,* (2002) especies *crípticas* son aquellas que son demasiado parecidas para haber sido separadas en base a su morfología mediante la taxonomía tradicional, pero que muestran diferencias de comportamiento, fisiológicas o de otra clase que resultan en un aislamiento reproductivo. Para Bickford *et al*., (2007) especies *crípticas* son dos o más especies que han sido clasificadas erróneamente (y escondidas) bajo un único nombre específico.

Son muchos los ejemplos de descripciones de nuevas especies a partir de lo que antes se consideraban como una sola, tal como ha ocurrido con *S. costatum* y *S. pseudocostatum*. Esto es más frecuente entre las especies tóxicas o potencialmente tóxicas ya que debido al evidente interés sanitario y económico de distinguir especies tóxicas de las no tóxicas, se ha invertido un mayor esfuerzo en los estudios taxonómicos y sistemáticos en los grupos con especies tóxicas.

De esta forma, recientemente se describió el *Azadinium polongum* (Tillmann *et al.*, 2012) en el que, tras constatarse que la células tenían diferencias genéticas que se consideraron significativas con *Azadinium spinosum*, se encontró una sutil diferencia en la forma de la placa Po, que en la primera especie es más larga que en la segunda, y en la ausencia de un pirenoide. Dado el pequeño tamaño de estas especies, no es fácil observar

estos caracteres, siendo necesario el uso del microscopio electrónico de barrido (SEM) para observar las diferencias en la forma de la placa Po, a lo que se añade que son especies muy difíciles de preparar para una observación adecuada en el SEM. En este caso se considera que estas especies son pseudocrípticas.

PROPUESTAS TAXONÓMICAS ACTUALES: TAXONOMÍA INTEGRADORA

En los últimos tiempos ha habido un progreso importante al reconocerse la distinción entre lo que son las especies, o los conceptos de especie, y las evidencias o criterios para reconocerlas y delimitarlas (Wiens, 2007, Yeates *et al.*, 2011). Este hecho ha revelado que parece que actualmente hay un acuerdo general de que las especies son linajes considerando éstos como series de metapoblaciones de ancestros y de sus descendientes (Padial et al., 2010, de Queiroz, 1998).

Sigue pues completamente actual lo que Darwin (1859) dijo al comienzo del capítulo II del *Origin*: «*Nor shall I here discuss the various definitions which have been given of the term species. No one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species*». Es entonces el momento de centrarse en cómo delimitar esos linajes.

Sites & Marshall (2004) hacen una revisión de 12 criterios operacionales para delimitar especies. Distinguen entre aquellos en los que se da prioridad al flujo genético, utilizados por biólolgos de poblaciones, y los basados en árboles en los que se busca la capacidad de distinguir linajes, que son los preferidos por los sistemáticos. Concluyen que todos los métodos fallan a veces a la hora de delimitar correctamente las fronteras entre especies, y que prácticamente todos obligarán a que los investigadores hagan juicios cualitativos. Por ejemplo, no hay ningún criterio objetivo sobre cuánta divergencia morfológica es suficiente para delimitar una especie aunque tradicionalmente, las especies se han identificado y descrito en base a su morfología. Los caracteres morfológicos a menudo están sometidos a procesos de evolución convergente, ya que pueden están sujetos a la misma presión selectiva. Los caracteres morfológicos también pueden estar indluenciados por factores no hereditarios como por ejemplo los ambientales. Un caso llamativo es el de *Ceratium ranipes*, una especie que se caracteriza por tener unas prolongaciones en forma de dedos al final de los cuernos pero que solo los tiene durante el día pues los pierde por la noche (Pizay et al 2009). El uso únicamente de datos morfológicos puede, por lo tanto, alterar el número de especies y, en particular, puede fallar para identificar especies crípticas. Los datos genéticos moleculares pueden proporcionar información adicional acerca de muchos factores relacionados con la identificación de especies, incluyendo identidades de poblaciones, niveles de flujos de genes recientes o antiguos, grados de hibridación, y relaciones filogenéticas entre

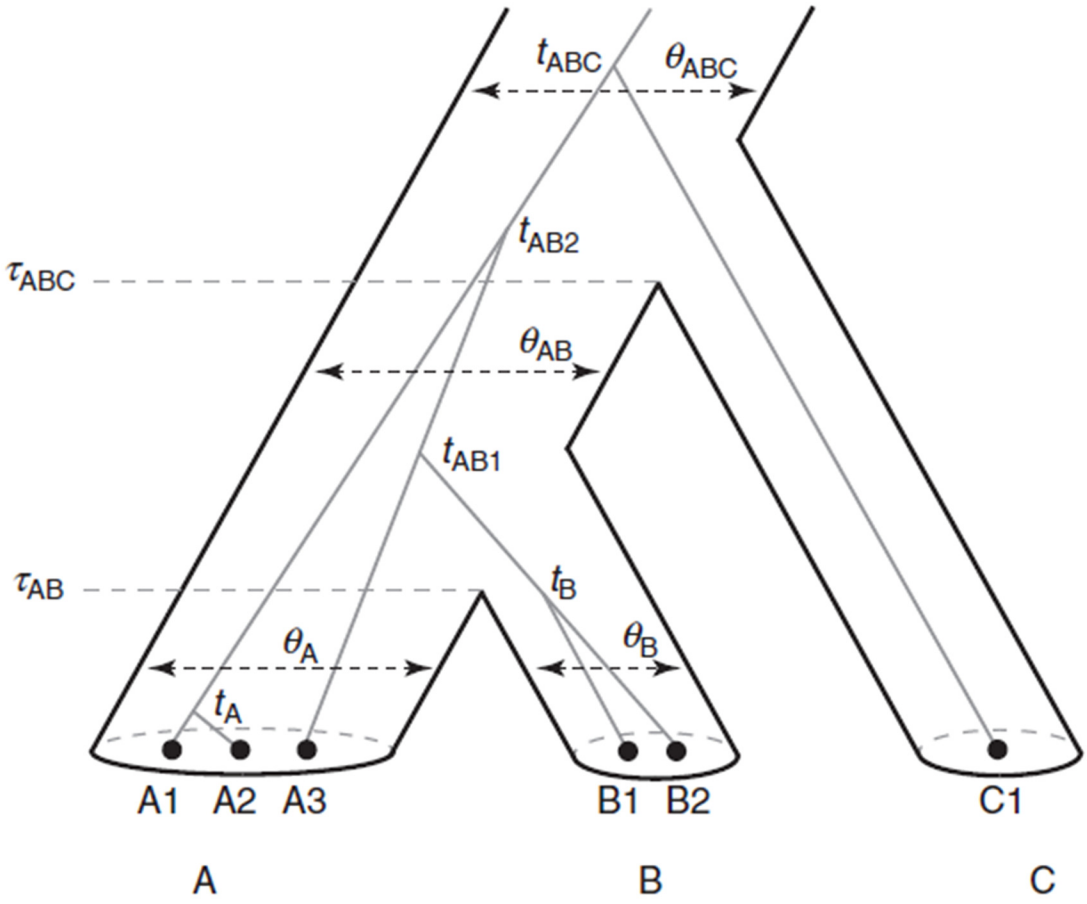

Figura 4. Coalescencia de múltiples especies y parámetros asociados utilizados en los modelos de delimitación de especies basada en coalescentes. Las ramas en negrita representan linajes de organismos de un arbol de especies (con las especies A, B y C). Los anchos corresponden al tamaño efectivo de las poblaciiones, y los nodos corresponden al momento de la especiación (t). El árbol gris sólido dentro del árbol de especies es un árbol de un único gen en el que los nodos corresponden a la coalescencia tiempos de alelos en la población (t). Nótese que el árbol de genes es diferente al árbol de especies (Adaptado de Fujita *et al*., 2012).

potenciales especies. Los modelos de inferencia filogenética y genética de poblaciones están cada vez más integrados que nunca (Edwards, 2009), y los modelos de delimitación de especies se benefician de ello. La teoría coalescente proporciona un marco teórico para modelar la historia de poblaciones de modelado, y el modelo coalescente multiespecífico (Ramala & Yang, 2003) se usa cada vez más para en métodos filogenéticos y de delimitación especies. El modelo coalescente multiespecífico rastrea la historia

genealógica de las muestras hasta hallar un ancestro común para las muestras que representan a múltiples especies (Fujita *et al.*, 2012) (Fig. 4)

Como ya se dijo más arriba, se estima que quedan muchos millones de especies por descubrir y describir y esto supone un gran reto. Hebert *et al*. (2003) consideran que, aunque la investigación biológica depende de los diagnósticos de las especies, la competencia taxonómica está desapareciendo y están convencidos de que la única posibilidad de mantener una capacidad de identificación sostenible radica en la construcción de sistemas que empleen secuencias de ADN como códigos de barras («Barcodes»). Ellos proponen el establecimiento del gen mitocondrial citocromo c oxidasa I (COI) como el núcleo de un sistema de bioidentification global para animales. Esta propuesta tiene muchas críticas al estar basada únicamente en una secuencia genética. Will *et al.* (2005) hacen un furibundo ataque al «barcoding» como substituto de la taxonomía normal y proponen el uso de todos los recursos disponibles para crear una capacidad real para haer el trabajo, y no únicamente el uso de una secuencia genética. Acuñan para esto el término de «taxonomía integradora», que se basa en el uso de un gran número de caracteres incluyendo DNA y muchos otros tipos de datos, para delimitar, descubrir e identificar especies naturales y con sentido, así como taxa a todos los niveles. Estos autores dejan claro que el debate acerca del «barcoding» no es el de DNA frente a morfología, sino el de una sistemática basada en un solo carácter, por ejemplo un gen, frente a una sistemática integradora basada en múltiples caracteres, incluyendo el DNA.

De una forma independiente, Dayrat (2005) propone el mismo término de «taxonomía integradora» casi simultáneamente que Will *et al*., (2005) y lo define como: '*Integrative taxonomy' is defined as the science that aims to delimit the units of life's diversity from multiple and complementary perspectives (phylogeography, comparative morphology, population genetics, ecology, development, behaviour, etc.)* y utilizó este término para postular un conjunto de directrices que tenían como objetivo facilitar la integración de datos de diferentes fuentes y que los taxónomos deben seguir al proponer nombres de especies. Las directrices propuestas por Dayrat son:

1. No se deben crear nuevos nombres de especies en un determinado grupo a menos que exista una revisión taxonómica reciente que se haya ocupado de la totalidad de los nombres disponibles para el grupo.

2. No se deben crear nuevos nombres de especies, si no se han abordado a fondo las variaciones infra e interespecíficas de los caracteres.

3 . No se deben crear nuevos nombres de especies utilizando menos de un cierto número de ejemplares (a determinar para cada grupo), y nunca con un único especimen.

4 . Un conjunto de especímenes que difieren en cierto modo de las especies existentes, se puede describir con la abreviatura «sp.» en lugar de un nombre de especie regulado por los códigos de la nomenclatura. Esta directriz enfatiza que la taxonomía integradora da prioridad a la delimitación de las especies, y no la creación de nuevos nombres.

5. Idealmente, sólo deben crearse nombres para especies que estén ampliamente apoyados por evidencias biológicas (morfología, concordancia genealógica, ecología, comportamiento, etc.).

6. No se deben crear nuevos nombres de especies, si los especímenes tipo depositados en una colección de museo se conservan de tal forma que impida cualquier posterior estudio molecular.

7. A partir de ahora, todos los neotipos designados deben preservarse de tal modo que permita extracciones de DNA y su secuenciación.

La propuesta de Dayrat (2005) no está exenta de críticas (Valdecasas *et al.*, 2008) pero, en general las ideas de Will *et al*. (2005) y de Dayrat (2005), aunque no son realmente nuevas, están teniendo gran aceptación. En taxonomía, el trabajo que se basa en múltiples fuentes y que se aprovecha de la complementariedad entre disciplinas, ha sido sido denominado de diversas formas: taxonomía combinada, multidisciplinaria, multidimensional, colaborativa, o integradora (Schlick-Steiner *et al.*, 2010) siendo este último término el que está siendo más aceptado últimamente. La taxonomía integradora usa un gran número de caracteres, incluyendo DNA y muchos otros tipos de datos, para delimitar, descubrir, e identificar especies y taxones a todos los niveles significativos y naturales aunque en muchos casos se centra en la especie. Está en el polo opuesto al «barcoding» no es ni será una solución ni un reemplazamiento de la ciencia sistemática. La descripción de taxones en base a un sistema de un solo carácter, ya sea morfológico o de un solo gen, será deficiente si no está en un contexto adecuado. La colaboración entre disciplinas como la filogeografía, anatomía comparada, la genética de poblaciones, la ecología y la biología del comportamiento debería convertirse práctica habitual en materia de taxonomía (Dayrat, 2005).

La taxonomía morfológica falla en algunos casos en los que es imperativo aplicar otros enfoques, pero incluso en los casos que ésta delimita especies con éxito, la ayuda de otros enfoques puede ser muy útil y acelarar todo el proceso. Además el uso de otras disciplinas ayuda a la taxonomía a ir más allá de darle nombre a las especies y entender los procesos que dan lugar a ellas. La taxonomía integradora está permitiendo disminuir en algunos casos el número de especies al descubrir sinonimias, pero al mismo tiempo lo aumenta al permitir descubrir especies crípticas. (Schlick-Steiner *et al.*, 2010).

INTRODUCCIÓN

La creciente importancia de la taxonomía integradora se ha reflejado en la celebración de una sesión del Ocean Sciences Meeting 2014 en Hawaii titulada: «Integrative Taxonomy of Marine Animals: Progress, Prospects and Pitfalls» (Organizadores: A. Bucklin, D. Lindsay, T.T. Sutton, F.H. Sinnigery) que se presenta con las siguientes palabras que hago mías:

«The emerging field of integrative taxonomy is yielding understanding of the taxonomy, systematics, and biodiversity of marine animals. Molecular methods have yielded new insights and have enormous potential for accurate and consistent identification of species, characterization of species diversity, and near-real time monitoring and assessment of marine communities. It seems likely that taxonomy and phylogeny of some groups will be greatly revised with the addition of molecular characters, yet traditional morphologically - based approaches will not be replaced, only enhanced and augmented. Near-future prospects include sophisticated, powerful and integrated analysis of morphological, molecular, biochemical, ecological, and geographic data to delineate species and test species hypotheses. This session will examine a broad range of methodologies, provide overviews of recent results using diverse types of data, and encourage discussion of how best to meet the challenges of integrative taxonomy of marine animals».

PRINCIPALES CARACTERÍSTICAS DE LOS DINOFLAGELADOS

Los dinoflagelados son eucariotas unicelulares, es decir, protistas, provistos de dos flagelos diferentes, uno en forma de cinta ondulante que bate hacia la izquierda de la célula y otro en forma de látigo que bate hacia la parte posterior (Fig 5). Unos dinoflagelados pueden vivir como organismos fotoautótrofos y representan aproximadamente la mitad de los géneros, otros como mixótrofos, que significa que además de ser capaces de realizar la fotosíntesis también pueden actuar como heterótrofos incorporando materia orgánica o fagocitando otros organismos, desde bacterias a otras microalgas o incluso microzooplancton. Otros son simbiontes o incluso parásitos. La mayoría de los dinoflagelados son marinos pero también los hay de aguas salobres y de agua dulce. Pueden ser planctónicos pero también bentónicos viviendo sobre diversos sustratos tanto inertes, como rocas o epífitos sobre macroalgas. Los de vida libre, junto con las diatomeas son importantes componentes del fitoplancton y de de la red trófica en el ambiente marino. Están muy bien adaptados a sus diferentes ambientes debido a su larga existencia que data desde el comienzo de los eucariotas. Aunque debido a su diversidad y modos de vida hay formas muy diferentes, los dinoflagelados típicamente nadan propulsados mediante sus dos flagelos con un característico movimiento rotatorio que dió origen a su nombre, dinoflagelados, del griego *dini* (remolino), más *flagellum* (flagelo en latín), diferente del *dino* de dinosaurio que significa peligro en griego. Mediante

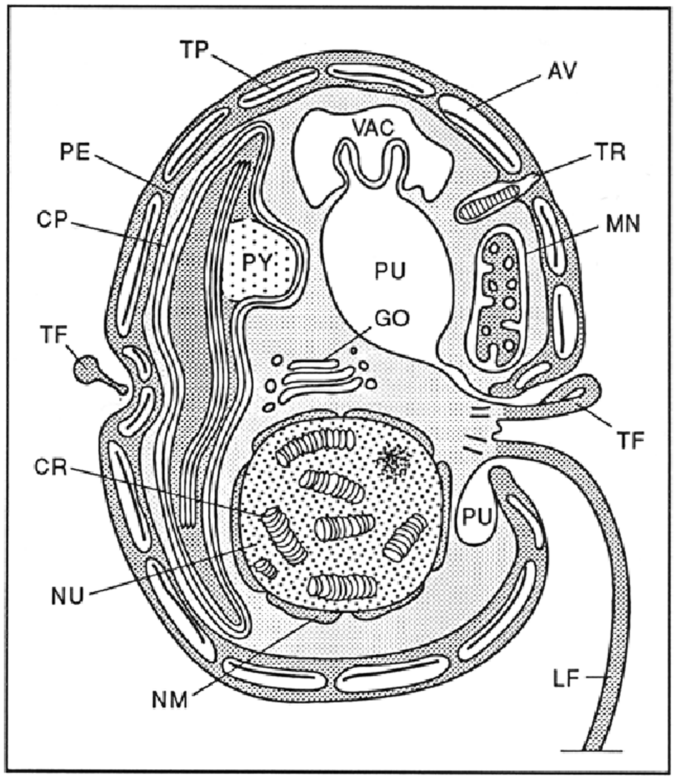

Figura 5. Sección generalizada de un dinoflagelado tecado móvil. AV = Vesículas anfiesmales, CP = Cloroplasto, CR = Cromosoma, GO = Aparato de Golgi, LF = Flagelo longitudinal, MN = mitocondria, NM = Membrana nuclear, NU = Núcleo, PE = Película, PU = Púsula, PY = Pirenoide, TV = Flagelo transversal, TP = Placa tecal, TR = Tricociste, VAC = Parte del vacuoma celular. (Adaptado de Fensome *et al* 1993, y éste, a su vez de Taylor, 1980)

esta capacidad natatoria, algunas especies pueden alcanzar velocidades considerables, y realizar migraciones verticales. Según sea la orientación de los flagelos se pueden hacer dos grupos: Aquellos que tienen la base de los dos flagelos en el lado hacia el que nadan, o sea el apical, son los desmocontes (por ejemplo, el género *Prorocentrum*) y aquellos que tienen su base en la parte ventral de tal forma que uno rodea la célula ecuatorialmente y otro es longitudinal y está generalmente dirigido hacia la parte antiapical, son los dinocontes, que son la mayoría. En esta memoria se trata únicamente de dinoflagelados dinocontes. La pared celular de los dinoflagelados es compleja y se denomina anfiesma. Tiene unas vesículas, lo que los incluye entre los protistas alveolados, y éstas pueden tener depósistos de celulosa de tal forma que consitituyen una armadura cuyas características morfológicas son ampliamente utilizadas para su identificación taxonómica. Según estas vesículas estén rellenas de celulosa o no, se pueden hacer dos grandes grupos,

el de los dinoflagelados desnudos y el de los tecados. Algunas, como las Suessiales tienen la vesículas con poca celulosa lo que las hace morfológicamente intermedias entre los dinoflagelados desnudos y los tecados, aunque no filogenéticamente. Muchas especies son bioluminiscentes, siendo la principal causa de la bioluminiscencia en las aguas marinas que en algunos casos es espectacular como ocurre en la Bahía Foforescente de Puerto Rico, donde *Pyrodinium bahamense* forma floraciones casi permanentes cuya bioluminiscencia constituye una atracción turística. Algunas especies son productoras de sustancias bioactivas entre las que abundan toxinas de muy diversos tipos que se tratarán en detalle más adelante. Los dinoflagelados, que en su fase vegetativa se considera que normalmente son haploides, pueden tener ciclos vitales muy complejos en los que se alternan fases haploides con fases diploides y fases planctónicas con fases bentónicas en forma de quistes. Hay gran variabilidad en la cantidad de DNA que poseen, pero algunas especies se encuentran entre los eukariotas con más cantidad de DNA por célula haploide. Tienen un núcleo muy peculiar, denominado dinocarion (Rizzo, 1991) que carece de histonas y presenta numerosos cromosomas permanentemente condensados. Entre los dinoflagelados fotosintéticos se pueden encontrar al menos seis tipos pigmentarios (Zapata *et al.*, 2012), sin que hasta este momento esté claro en todos los casos si se trata de cloroplastos que se puedan considerar como propios o como casos de cleptoplastidia.

DINOFLAGELADOS NOCIVOS

El concepto de nocividad de las microalgas es fundamentalmente antropocéntrico, por lo que cuando éstas se califican como nocivas es generalmente a causa de algún efecto que produzcan y que, desde el punto de vista humano, se considere nocivo, aunque éste sea un engranaje más del ecosistema. La posición trófica de la especie humana, actualmente, dista mucho de la de sus antecesores primates. Así, hoy en día nos alimentamos de muchos recursos marinos a los que nuestra especie, antiguamente, no tenía acceso. Algunos de esos recursos son peces o mariscos que portan sustancias que afectan a la salud de las personas cuando se ingieren. Este tipo de daño, el causado a la salud pública a través de la red trófica, es el de más importancia y el que acapara más atención por nuestra parte. La mayoría de los daños de este tipo son intoxicaciones causadas por la ingestión de moluscos filtradores que han acumulado sustancias producidas por determinadas microalgas y que para nosotros son tóxicas y en algunos casos fatales, o por la ingestión de peces que han acumulado toxinas producidas por las microalgas también a través de la red trófica.

En el caso de las intoxicaciones a través de moluscos filtradores se pueden distinguir también varios tipos según los distintos venenos que las produzcan y que son de muy

diversa estructura y origen, y por lo tanto causantes también de diversos síndromes. El que más atención ha recibido tal vez sea el de tipo paralizante conocido en España por sus siglas inglesas PSP (Paralytic Shellfish Poisoning) y que está causado por varias especies de dinoflagelados del género *Alexandrium*, o por especies de otros géneros como *Gymnodinium catenatum* o *Pyrodinium bahamense*. La toxinas responsables son las del grupo de la saxitoxina, de las que se conocen muchos análogos según sean algunos de los radicales, siendo su toxicidad diferente de unas a otras y también transformables a lo largo de sus síntesis biológica o del metabolismo de los organismos que las ingieran. La composición del complejo de toxinas es variable de unas especies a otras y en ciertos casos puede considerarse como una huella digital que permite identificar las especies causantes de una intoxicación por su perfil de toxinas. Otro grupo de intoxicaciones que tienen como síntoma más llamativo la diarrea se conocen con las siglas DSP (Diarrhetic Shellfish Poisoning) y lo causan principlamente toxinas del grupo del ácido okadaico, una toxina del grupo de los poliéteres producida por diversas especies de dinoflagelados de los géneros *Dinophysis* y *Prorocentrum*. Debido a síntomas parecidos, a sus afinidades químicas y detección mediante el bioensayo del ratón, hay otras toxinas que algunos autores incluyen dentro del DSP pero que podrían incluirse en un grupo más grande de toxinas liposolubles, al contrario de la saxitoxina y el ácido domoico que son hidrosolubles. Estas toxinas son las pectenotoxinas, producidas por *Dinophysis*, los azaspirázidos, detectados en dinoflagelados del género *Azadinium* y las yesotoxinas, producidas por dinoflagelados de diversos géneros como son *Protoceratium reticulatum*, *Lingulodinium polyedrum* o *Gonyaulax spinifera*. Las gymnodiminas son un grupo de toxinas producidas por *Karenia selliformis* que la transmite a moluscos filtradores.

Las intoxicaciones a través de la red trófica pueden ser tambien causadas por la ingestión de peces. El síndrome más conocido en este caso es la ciguatera que es una enfermedad propia de aguas tropicales que afecta a muchos miles de personas anualmente principalmente en áreas del Caribe y de la Polinesia. Las principales toxinas causantes de este síndrome son las ciguatoxinas, unos derivados de otras producidas por dinoflagelados bentónicos del género *Gambierdiscus* que crecen sobre macroalgas, rocas o arenas. Entran en la cadena trófica a través de peces herbívoros que ramonean las algas de arrecifes y que las acumulan y transforman en sus tejidos. Luego se transmiten a peces carnívoros acumulándose en cada eslabón como si se tratase de un metal pesado de tal forma que los peces de niveles tróficamente altos como la barracuda o la morena, son los que suelen mostrar índices más altos de toxicidad. Los *Gambierdiscus* crecen generalmente junto a otros dinoflagelados tóxicos de diversas especies como de *Prorocentrum* que producen toxinas del grupo del ácido okadaico o de *Ostreopsis* que producen palitoxinas. No puede descartarse que estas toxinas tengan pues alguna participación en la ciguatera pues los síntomas de ésta son bastante variables y, a veces, imprecisos y esta variabilidad podría ser debida a diferentes composiciones de este «cocktail» de toxinas. Los *Gambierdiscus*

pueden sintetizar tambien otra toxina muy potente, la maitotoxina, aunque ésta apenas se ha encontrado en peces ciguatos. Las palitoxinas producidas por *Ostreopsis* también son muy potentes y aparte del papel que puedan jugar en la ciguatera, han causado algunas muertes a través de peces o erizos, y tal vez sean las responsables de algunos daños directos a la salud causando problemas respiratorios en bañistas. Este tipo de daños lo causan otros dinoflagelados del género *Karenia* y actualmente están en estudio algunos casos ocurridos en el Mediterráneo y coincidentes con grandes concentraciones de *Ostreopsis* en las aguas.

Otras veces las microalgas causan daños a otros organismos marinos, aunque la idea de daño tambien en este caso está fuertemente impregnada de antropocentrismo. Las mortandades de algunas especies a causa de otras son parte de la sucesión ecológica y entran dentro del dinamismo de los ecosistemas. Así las mortandades masivas de peces silvestres están registradas desde antiguo. Donde el antropocentrismo está claro es en aquellos casos en los que las víctimas son peces en cultivo. Si en algún mar del mundo en el que se cultiven peces en cautividad no ha habido mortandades masivas probablemente sea porque todavía llevan poco tiempo. Cuando hay una prolifereación de alguna especie que puede causar daño a los peces, éstos libremente se desplazan a zonas en las que no se encuentren esas algas. Sin embargo, si están en jaulas, no podrán evitarlos y entonces resultan afectados. Las mortandades de peces pueden ser causadas por especies de diversos grupos de microalgas, como rafidofíceas o haptofitas, pero en la mayoría de los casos, son dinoflagelados los responsables. Si se dan proliferaciones con biomasas elevadas, cuando éstas decaen hacen descender los niveles de oxígeno hasta niveles muy bajos que pueden ser letales para algunos organismos. En otros casos, la asfixia se produce por taponamiento de las branquias con mucus o causándole una irritación que impide que ejerzan su función. También pueden producir mortandades por la toxinas, siendo el caso más frecuente el de especies de los géneros *Karenia* y *Karlodinium*.

El caracter dañino más «humano» es a la economía. Es obvio que cualquier daño que afecte a la salud pública a través de la acuicultura o de productos pesqueros, afecta seriamente a estos sectores, pero un aspecto que cada día tiene mayor importancia es el turístico. Si playas o calas, famosas por la transparencia de sus aguas se ven afectadas por floraciones de microalgas que las hacen turbias o que las llenan de mucílagos, se registran serios daños al sector turístico.

CRITERIOS UTILIZADOS PARA TAXONOMÍA DE DINOFLAGELADOS.

Hasta principios de los años noventa, la clasificación de los dinoflagelados, tanto la de los taxones vivientes como los fósiles, se basaba casi exclusivamente en características morfológicas (Taylor, 2004b, Taylor, 2004a). Cuando, en el caso de los dinoflagelados

tecados era posible determinar la tabulación de las placas, esto se consideraba de primordial importancia ya que se sabía mucho sobre cuales eran los caracteres más o menos conservativos del mismo modo de como la clasificación de diatomeas presta gran importancia a pequeños detalles de poros en sus frústulos. Los grupos de dinoflagelados atecados o desnudos se consideraban polifiléticos, pero debido a la falta de herramientas suficientes permanecían poco estudiados. Con la llegada de los estudios moleculares, el número de especies nuevas de dinoflagelados desnudos se ha disparado en los últimos años, mientras que entre los dinoflagelados tecados no es tan acusado. Los paleontólogos han estudiado los quistes fósiles de los dinoflagelados sin que hasta los años sesenta se pudiesen considerar como tales (Wall & Dale, 1966). En esa época se comienza el estudio de los quistes de dinoflagelados actuales, estudio que hasta ese momento estaba reservado a los fósiles de lo que se consideraban grupos extintos. Las ornamentaciones de los quistes (espinas, aletas,etc) y la forma del arqueopilo constiutían la base de la clasificación de los dinoflagelados fósiles, de tal forma que se creó para los dinoflagelados fósiles una sistemática independiente de los taxones vivientes basada en las placas (Sarjeant & Downie, 1966). La unión de los conocimientos sobre los quistes fósiles a los de los dinoflagelados actuales, dió lugar a discusiones, en las que para resaltar estas diferencias se hablaba jocosamente, en inglés, de «Systematics» contra «Cystematics».

La filogenia de los dinoflagelados es compleja y en ella son frecuentes los casos de transferencia horizontal de genes. Los diversos grupos pigmentarios de los dinoflagelados (Zapata et al., 2012) son el producto de una serie de endosimbiosis que han ocurrido a lo largo de la evolución de los protistas (Falkowski *et al.*, 2004) . Por esta razón, el estudio de los pigmentos es una pieza importante en la taxonomía de los dinoflagelados.

La taxonomía de los dinoflagelados se ha basado en la morfología y bajo el criterio personal de cada taxónomo haciendo muy cierto el concepto morfológico de especie de Regan que se ha citado más arriba: *«Una especie es una comunidad, o un número de comunidades relacionadas, cuyos caracteres morfológicos distintivos están, en la opinión de un sistemático competente, suficientemente definidos como para denominarla o denominarlas con un nombre específico».* (Regan, 1926: 75, citado en Mayden (1997)) [A la palabra «comunidad» no se le da aquí el significado que actualmente tiene en ecología]. Algunos legendarios «sistemáticos competentes» marcaron las pautas para el desarrollo de la taxonomía morfológica y muchos de sus criterios siguen en uso aunque ya estén superados.

En esta tesis se trata solamente de dinoflagelados dinocontes y, aunque no tenga caracter sistemático alguno, por razones prácticas de su estudio, éstos se pueden dividir en tecados y desnudos. Por convenio, la parte de la célula hacia donde nada la célula, se denomina anterior, el lado del que parten los flagelos, el ventral, definiéndose a partir de estas, la parte posterior y la dorsal. Los dinoflagelados dinocontes tienen dos partes

separadas por el cíngulo en el que se aloja el flagelo transversal, la anterior se llama epiteca en los tecados y epicono en los desnudos y una posterior, en la que generalmente hay un surco logitudinal que aloja el flagelo lonfitudinal que es la hipoteca o hipocono.

Dinoflagelados desnudos

La taxonomía de los dinoflagelados desnudos, antes de la aparición de las técnicas moleculares, se basó en características tales como el tamaño y contorno de la célula, forma del epicono y del hipocono, la posición relativa del cíngulo, el desplazamiento cingular y si las células presentaban torsión o no. De esta forma se definieron los géneros *Amphidinium*, *Gymnodinium* y *Katodinium*, según la posición del cíngulo fuese anterior, ecuatorial o posterior respectivamente, o *Gyrodinium* con cíngulo ecuatorial pero que se distinguía de *Gymnodinium* en que tenía un desplazamiento cingular de más de 3/5 del total de la longitud de la célula. Si las células presentaban una torsión de tal forma que el cíngulo daba más de una vuelta a la célula, era *Cochlodinium*. Otros géneros se basaban en otras características tales como la presencia de mánchas oculares, pedúnculos, etc. Dentro de cada célula, las diferentes especies se distinguían por la forma general de la célula, por si tenían estrías o no, etc. (Kofoid & Swezy, 1921). Con la llegada de las técnicas moleculares se comenzó una revisión de toda la taxonomía de los dinoflagelados desnudos con la redefinición de los principales géneros y erección de otros nuevos en base a no solo la morfología general, sino a la genética, composición pigmentaria, y ultraestructura (Daugbjerg *et al.*, 2000). Entre las características que ahora se consideran importantes, está la composición pigmentaria. En primer lugar hay que distinguir aquellos que son heterótrofos, como *Gyrodinium* de los autótrofos. La mayoría de los dinoflagelados fotosintéticos tienen cloroplastos con peridinina como principal carotenoide, pero se han detectado cloroplastos de otros grupos de algas, lo que sugiere múltiples pérdidas de plastidios y substituciones a través de procesos de endosimbiosis. En la base a combinaciones únicas de clorofilas y carotenoides, Zapata et al (2012) definieron 6 tipos pigmentarios entre los dinoflagelados de los cuales 4 están formados por dinoflagelados desnudos. Además de la clorofila a, la mayoría tienen peridinina, dinoxantina y clorofila c2 como principales pigmentos, pero otro grupo tiene clorofila b junto con neoxantina y violaxantina (*Lepidodinium chlorophorum*), otros dos, derivados de la fucoxantina (géneros *Karenia* y *Karlodinium*). Mientras que los plastos con peridinina, y probablemente los que tienen clorofila b, son fruto de procesos de endosimbiosis secundarias, los otros tipos de cloroplastos se obtuvieron a través de la endosimbiosis terciarias de haptofitas. Otras características importantes son la posición del núcleo, la presencia o no de pirenoides y la forma y número de los cloroplastos si los tienen. En algunos casos se ha usado la estructura del aparato flagelar, pero debido a la dificultad de su estudio son pocos los autores que lo usan. En relación a la morfología externa observable con microscopía

electrónica de barrido (SEM), la forma del surco apical o acrobase es un carácter que permite la diferenciación entre géneros. Por ejemplo, en *Gymnodinum* tiene forma de herradura, en *Karenia* y *Karlodinium* de surco recto que va de la parte ventral a la dorsal, en *Akasiwo* y *Barrufeta* distintas formas de lazo. En algunas especies se pueden distinguir las vesículas poligonales del amfiesma, que a veces se ven reflejadas en sus quistes de resistencia.

Dinoflagelados tecados

La taxonomía de los dinoflagelados tecados se basa fundamentalmente en el número, forma y disposición de las placa tecales aunque también se utilizan criterios ultraestructurales y pigmentarios de los usados con los dinoflagelados desnudos. Aparte de aquellos géneros en los que la peridinina es el pigmento mayoritario, entre los tecados hay otros dos grupos pigmentarios: Uno que tiene pigmentos de diatomea como resultado de una endosimbiosis que, en algunos casos todavía no se ha completado pues todavía quedan restos del núcleo de diatomea, como en *Kryptoperidinium foliaceum* (Figueroa *et al*., 2009) y otros que tienen cloroplastos y pigmentos de criptofitas y en los que la endosimbiosis todavía está más atrasada pues tienen que incorporar cloroplastos frescos a traves de su alimentación, como ocurre en *Dinophysis* (Rial *et al*., 2013).

Para el estudio de las placas, Kofoid (1909) desarrolló un sistema de nomenclatura de las placas en base a que éstas se disponen en series ecuatoriales (Fig. 6). Dentro de cada serie, las placas se numeran, a partir de la parte ventral, hacia la izquierda, o lo que es lo mismo, en visión apical, en sentido contrario a las agujas del reloj. A aquellas placas que están en contacto con la placa del poro apical, les llama apicales y se nombran numeradas con una comilla. Las de la serie previa al cíngulo, son las precingulares y se nombran con números con dos comillas. Aquellas que pueda haber entre las dos series, son intercalares anteriores y se denominan con un número seguido de la letra «a». Las cingulares se numeran con el prefijo de la letra «c». Las inmediatamente después del cíngulo, son las postcingulares y se numeran con tres comillas. Las antiapicales con cuatro comillas, y las que estén entre las postcigulares y las antiapicales intercalares posteriores y se numeran seguidas de la letra «p». El sulcus está formado por un número variable de placas comprendidas entre la más anterior, la sulcal anterior (S.a) y la sulcal posterior (S.p) que se denominan como sulcal anterior derecha (S.d.a) y sulcal anterior izquierda (S.s.a), sulcal posterior derecha (S.d.p) y sulcal posterior izquierda (S.s.p). Puede haber otras pequeñas placas. Según el número de placas de cada serie se obtiene la fórmula tecal tal como Po, 4', 3a, 6'', 3c, ?s, 5''', 0p, 2'''', que sería de un dinoflagelado que tuviese 4 apicales, 3 intercalares anteriores, 6 precingulares, 3 cingulares, un número indeterminado de sulcales, 5 postcingulares, ninguna intercalar posterior y dos antiapicales.

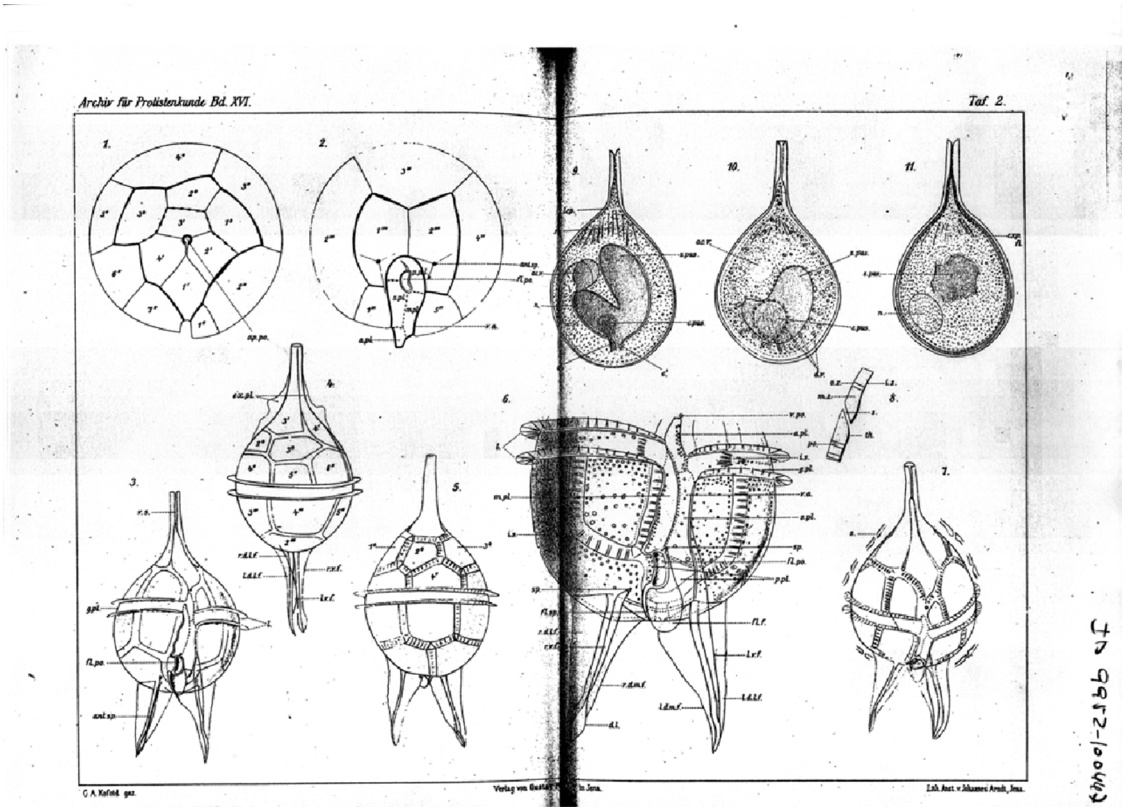

Figura 6. Ilustración de Kofoid (1909) donde éste muestra su original sistema de nomenclatura de placas de dinoflagelados tecados.

El sistema de Kofoid es muy útil para denominar las placas de estos puzles, pero no tiene en cuenta la homología de placas entre géneros (Eaton, 1980) o incluso entre especies del mismo género. Las placas son un elemento fenotípico sujeto a evolución, por lo que hay homologías de placas entre géneros y que en algunos casos aparezcan dos placas cuya suma sería la homóloga de una sola placa en otro género. Aunque hay muchos géneros que comparten fórmula tecal, siempre se ha considerado que dos fórmulas tecales diferentes corresponden a dos géneros diferentes. Ocurre que si en un determinado organismo hay dudas en la clasificación de una placa, al colocar ésta en una serie diferente a la de especies afines, ésto sería razón suficiente para colocarla en otro género. Un ejemplo muy importante de un caso de este tipo, lo constituye la transferencia de muchas especies del género *Gonyaulax* al género *Alexandrium* (Balech, 1985, Balech & Tangen, 1985). Dentro del género *Gonyaulax*, durante mucho tiempo se consideró que había un grupo de especies con características comunes tales como falta de espinas, forma redondeada y teca lisa, que las diferenciaba del resto de las especies del género, por lo

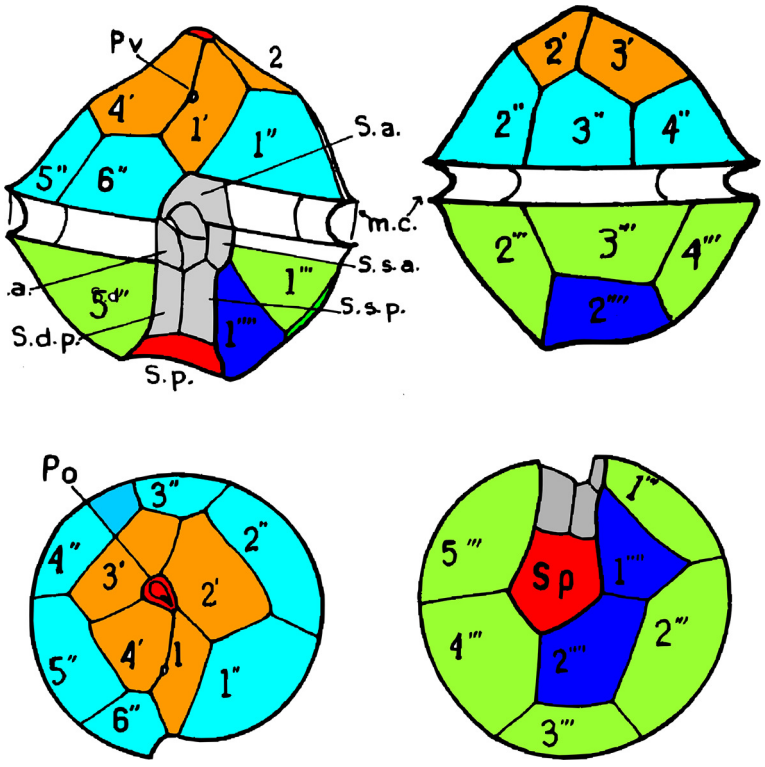

Figura 7 Tabulación y nomenclatura en *Alexandrium* siguiendo el criterio de Balech (1995). En ambar, serie apical; en azul, serie precingular; en verde, serie postcingular; en azul oscuro, serie antiapical; en rojo, sulcal posterior.

que aylor (1979) creó el género *Protogonyaulax* al que transfirió esas especies. Dejó sin embargo, fuera de este género al *Alexandrium minutum* Halim (1960), muy parecido a las especies transferidas al género *Protogonyaulax* pues en su descripción original tenía tres placas apicales y siete precingulares, mientras que *Protogonyaulax* tenía cuatro apicales y seis precingulares. El problema radicaba en que la 1' de *Protogonyaulax* era la placa homóloga de la 1'' de *Alexandrium.* Balech & Tangen (1985) consideraron que el caracter de contacto entre esa placa y la Po era algo variable y dudoso en algunas especies, como ya había mostrado Balech (1979) en la descripción de *Gonyaulax kutnerae*, por lo que considerando que no era un caracter como para separar dos géneros, optaron por transferir varias especies de *Gonyaulax* a *Alexandrium*, operación que completó Balech en otro trabajo (Balech, 1985) sin aceptar el género *Protogonyaulax* propuesto por Taylor ya que el género creado por Halim (Halim, 1960) tenía prioridad por ser más antiguo. Tras una discusión durante la *Third International Conference on Toxic Dinoflagellates* celebrada en Canadá en 1985, de Enrique Balech con F.J.R. «Max» Taylor y Yasuwo Fukuyo, que había adoptado el género *Protogonyaulax*, Balech se vió obligado a revisar material de *A.*

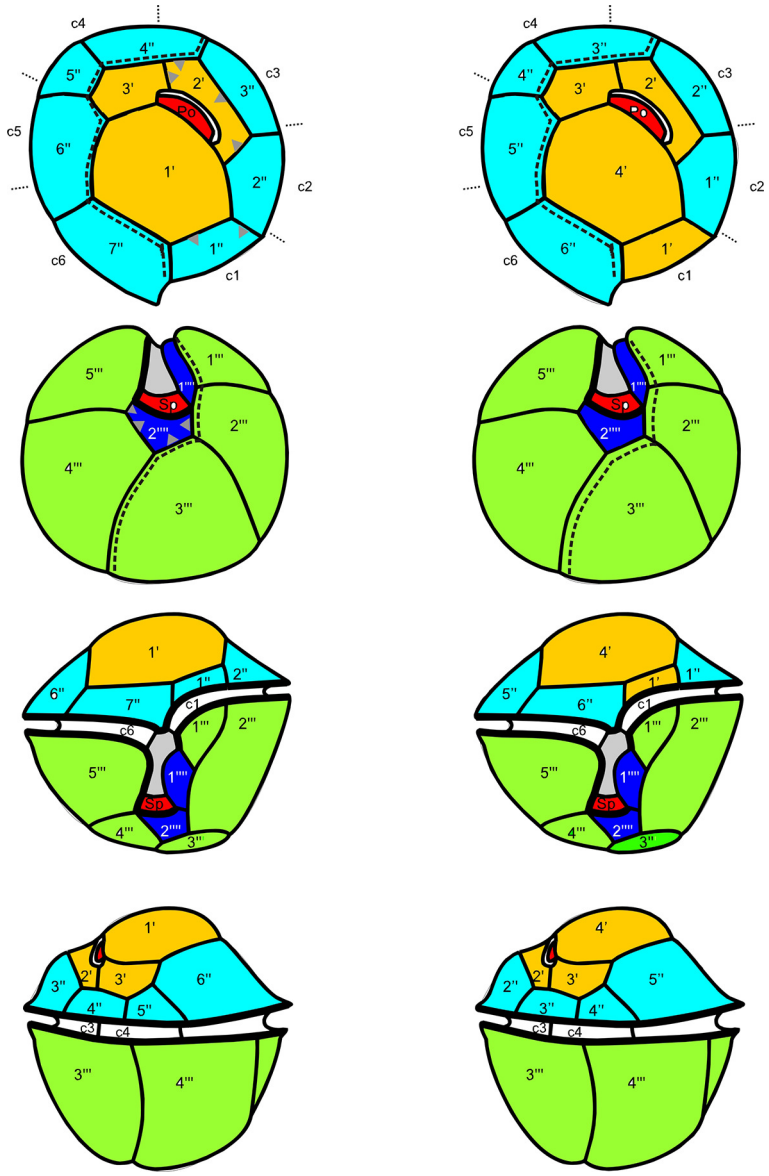

Figura 8. Tabulación en *Coolia*. A la izquierda modelo tgradicional kofoidiano.. A la derecha, modelo teniendo en cuenta las homologías de placas. En ambar, serie apical; en azul, serie precingular; en verde, serie postcingular; en azul oscuro, serie antiapical; en rojo, sulcal posterior.

minutum, obtenido de su localidad tipo en Alejandría, Egipto. Balech recibió el material de Halim y que él aceptó como *A. minutum* y con él llevó a cabo una cuidadosa redescripción de la especie tipo del género *Alexandrium* (Balech, 1989) en la que mostraba

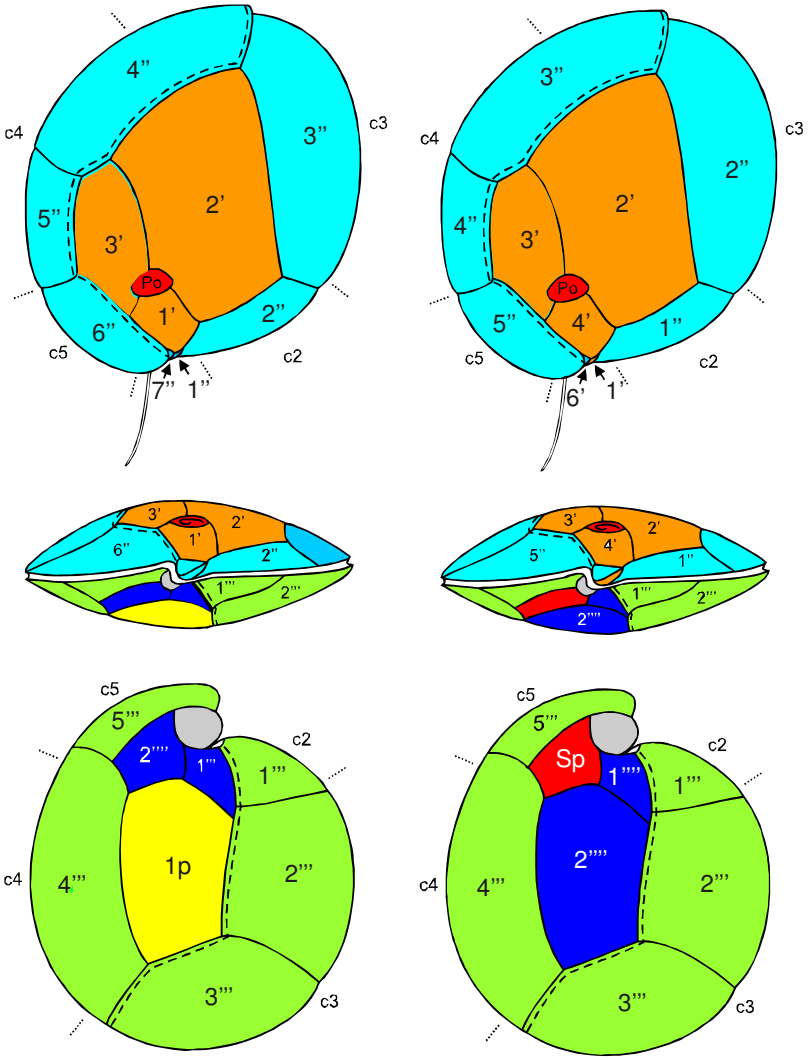

Figura 9. Tabulación en *Gambierdiscus*. A la izquierda modelo tgradicional kofoidiano.. A la derecha, modelo teniendo en cuenta las homologías de placas. En ambar, serie apical; en azul, serie precingular; en verde, serie postcingular; en azul oscuro, serie antiapical; en rojo, sulcal posterior.

que la placa 1’ podía estar en estrecho contacto con Po, unida por un filamento, o claramente separada, por lo que este contacto o su falta no podría ser un caracter para diferenciar géneros. En todas las especies que Taylor transfirió a *Protogonyaulax*, la placa 1’, además de tocar a Po también estaban en contacto con la sulcal anterior (S.a.) por lo que también podría se considerada como precingular. Entonces una vez clarificada esta homología entre placas, a la placa orígen de la discusión se pasó a llamársele siempre primera apical

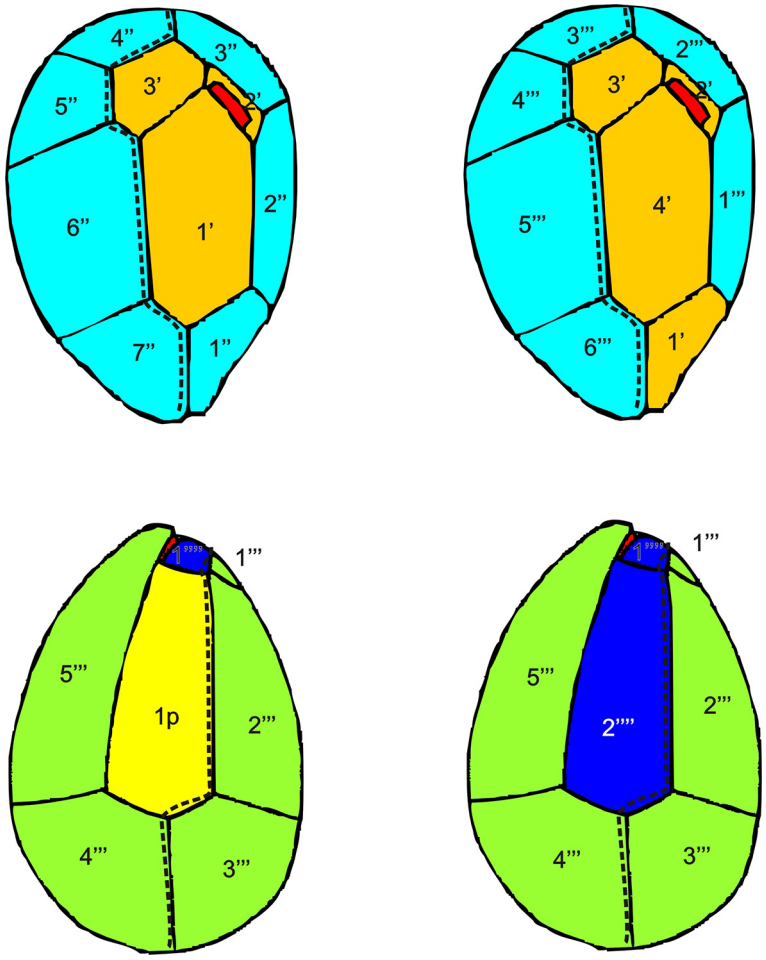

Figura 10. Tabulación en *Ostreopsis*. A la izquierda modelo tgradicional kofoidiano.. A la derecha, modelo teniendo en cuenta las homologías de placas. En ambar, serie apical; en azul, serie precingular; en verde, serie postcingular; en azul oscuro, serie antiapical; en rojo, sulcal posterior.

(1’) tocase o no a Po. Esto es una clara excepción al uso de la nomenclatura kofoidiana pero que facilitaba la interpretación de las placas.

Esto se utilizó en el género *Alexandrium* pero no en otras Gonyaulacales como *Coolia*, *Ostreopsis* o *Gambierdiscus* en las que la fórmula generalmente utilizada era la de tres apicales y siete precingulares, excepto en un detallado trabajo sobre morfología de estas especies en el Mar Caribe (Besada *et al.*, 1982) en el que en base a los solapamientos en las suturas de las placas y las lineas de fisión durante la división celular se inclinan por una nomenclatura de las placas como la propuesta por Balech para *Alexandrium*. En esta tesis, esta nomenclatura la hemos adoptado por primera vez en la publicación de

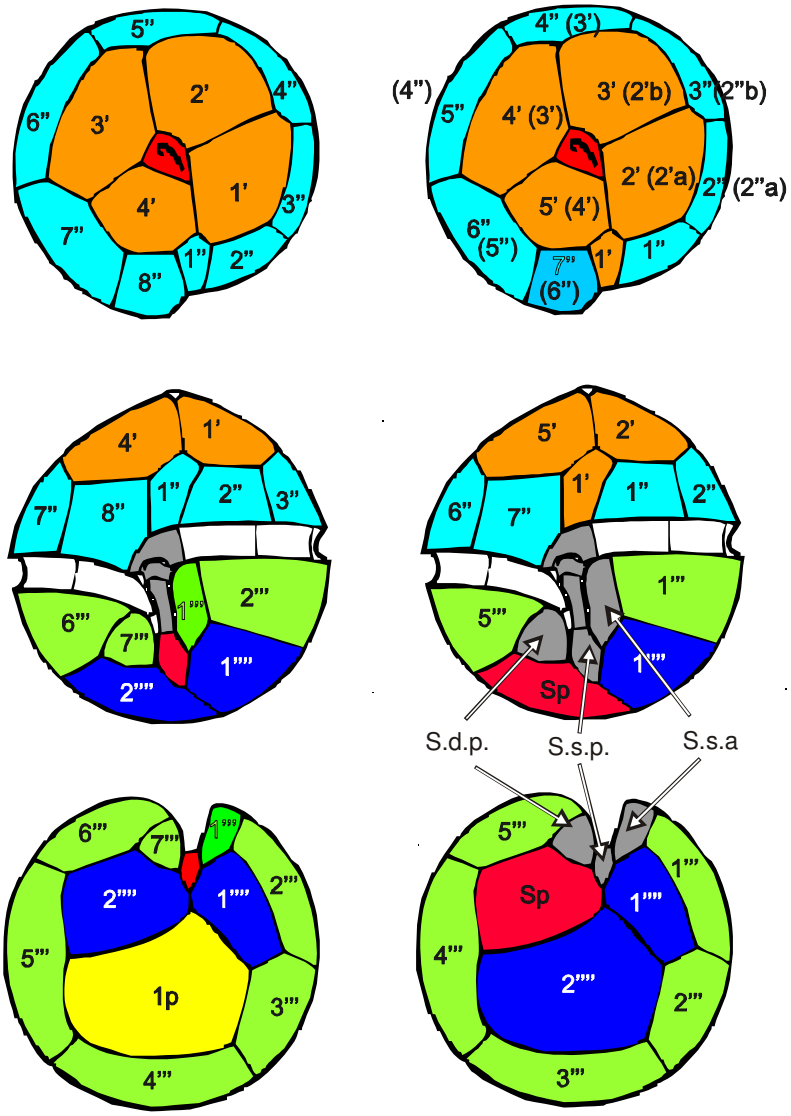

Figura 11. Tabulación en *Fragilidium*. A la izquierda modelo tgradicional kofoidiano.. A la derecha, modelo teniendo en cuenta las homologías de placas. En ambar, serie apical; en azul, serie precingular; en verde, serie postcingular; en azul oscuro, serie antiapical; en rojo, sulcal posterior.

Gambierdisucs excentricus (Fraga *et al.*, 2011), aunque en la publicación de *Coolia canariensis* (Fraga *et al.*, 2008) todavía se utilizó la generalizada en ese momento de tres apicales y siete precingulares. Sin embargo, en esta memoria ya se utiliza la nomenclatura de Besada (Figs. 8-10). Tras la publicación de la descripción de *G. excentricus*, esta nomenclatura ya está ampliamente aceptada de nuevo como se refleja en, por ejemplo la

descripción original de *Gambierdiscus scabrosus* (Nishimura et al., 2014). Aunque para algunos autores resulte chocante que la sulcal posterior esté fuera del sulcus en *Gambierdiscus*, no debe dársele más importancia al nombre dado a la placa que a su naturaleza. Un caso más extremo de la utilización de este criterio es en el género *Fragilidium* (Amorim *et al.*, 2013) en el que hemos encontrado que el contacto entre 1' y Po se da en los gametos pero no en los cigotos. En ese trabajo también hemos observado que, además de la S.p., otras placas sulcales, la S.d.p., la S.s.a y la S.s.p. están fuera del sulcus (Fig. 11) por o que otros autores consideraban que tenía 7 postcingulares en lugar de 5.

El problema sobre la nomenclatura de las placas no era nuevo cuando Besada *et al* (1982) publicaron su trabajo. Poco antes, cuando en 1978 se celebró Conferencia Penrose sobre dinoflagelados modernos y fósiles había dos cuestiones candentes: 1) La numeración Kofoidiana no puede expresar las homologías de las placas, y de hecho las oculta, y 2) algunos investigadores estaban aplicando estrictamente los criterios de Kofoid mientras que otros, como Besada *et al* (1982), los utilizaban de un modo laxo para tratar de expresar las homologías. En esa conferencia, F.J.R. «Max» Taylor y G.L. Eaton presentaron unas alternativas al sistema de numeración de Kofoid subrayando el reconocimiento de placas homólogas y basándose en un modelo con un alto grado de simetría (Edwards, 1990). Con posterioridad a la conferencia, se publicaron más alternativas al sistema de Kofoid (Edwards, 1990, Evitt, 1985, Taylor, 1979, Taylor, 1980) y luego el sistema de Taylor modificado por Evitt fué en parte utilizado por Fensome *et al* (1993) en su monográfica clasificación de los dinoflagelados vivos y fósiles. Aunque estos sistemas son mucho más lógicos que el de Kofoid, tal vez por la inercia en la utilización del sistema de Kofoid, que es muy intuitivo, actualmente la opción más común es la de utilizar la nomenclatura kofoidiana de un modo laxo para permitir las homologías como se hace en esta memoria.

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OBJETIVOS Y CONTENIDO DE LA TESIS

La creciente incidencia de los daños causados por dinoflagelados para la salud pública, la acuicultura, la pesca y el turismo han provocado un aumento en la necesidad de una mejor caracterización taxonómica de muchos dinoflagelados. La disparidad de las toxicidades y la ecología de algunas morfoespecies mostraron la posible presencia de especies crípticas donde hasta ese momento se consideraba que había una sola especie. Esta necesidad práctica de alcanzar una correcta identificación de las diferentes especies de dinoflagelados nocivos que permita distinguirlos de especies afines inocuas, es un motor que fuerza el avance en el desarrollo de nuevos criterios y herramientas para la caracterización taxonómica de esas especies y al cuestionamiento mismo de los conceptos de especie habitualmente utilizados en estudios ecológicos y toxicológicos de protistas. El objetivo principal de esta tesis es contribuir a la clarificación taxonómica de algunas especies de dinoflagelados nocivos y especies inocuas cercanas. En ella se aborda la resolución de problemas taxonómicos presentados por especies nocivas pertenecientes a los dos dos grandes grupos de dinoflagelados, los desnudos y los tecados. Del primer grupo se tratan dos especies de dinoflagelados desnudos muy similares y fácilmente confundibles, el tóxico *Gymnodinium catenatum* y el no tóxico *Gymnodinium impudicum*. Del segundo grupo, se estudian dinoflagelados bentónicos Gonyaulacales entre los que se encuentran productores de toxinas, de los géneros *Coolia* y *Gambierdiscus*, éste último al que pertenecen las especies que causan la ciguatera.

El estudio de estos grupos tan diversos de dinoflagelados pone de manifiesto las grandes diferencias ecológicas que se presentan entre ellos.

La mayor parte de esta tesis se basa en trabajos ya publicados en diversas revistas o en presentaciones en congresos.

La parte de la tesis relativa al género *Gymnodinium* se basa en los siguientes artículos:

Estrada, M., F.J. Sánchez y **S. Fraga.** (1984). *Gymnodinium catenatum* Graham en las rías gallegas (NO de España). Investigación Pesquera, 48(1): 31-40. (Primera cita de *G. catenatum* en el Atlántico Norte)

Fraga, S., I. Bravo, M. Delgado, J.M. Franco, Y M. Zapata (1995) Differences between two chain forming, athecate, red tide dinoflagellates: *Gymnodinium catenatum* Graham and *Gyrodinium* sp. En: *Harmful Marine Algal Blooms*. Lassus et al. eds. Lavoisier, París, pp.39-44. (Se destacan las diferencias entre *Gymnodinium catenatum* y el que posteriormente se describió como *Gyrodinium impudicum*)

Fraga, S., I. Bravo, M. Delgado, J.M. Franco y M. Zapata (1995). *Gyrodinium impudicum* sp. nov. (Dinophyceae), a non toxic, catenate, red tide dinoflagellate. *Phycologia*, 34(6): 514-521. (Descripción de una especie nueva diferente de *G. catenatum*)

La parte de la tesis relativa a las Gonyaulacales bentónicas se basa en los siguientes artículos:

Fraga, S, Penna, A, Bianconi, I, Paz, B, Zapata, M, (2008) *Coolia canariensis* sp. Nov. (Dinophyceae), a new non toxic epiphytic benthic dinoflagellate from the Canary Islands. *Journal of Phycology*, 44 (4) : 1060-1070. (Se describe una nueva especie de *Coolia*).

Mohammad-Noor, N., Moestrup, Ø., Lundholm, N., **Fraga, S.**, Adam, A., Holmes, M. J. & Saleh, E. 2013. Autecology and phylogeny of *Coolia tropicalis* and *Coolia malayensis* (Dinophyceae), with emphasis on taxonomy of *Coolia tropicalis* based on light microscopy, scanning electron microscopy and LSU rDNA. *J Phycol* 49:536–45. (Redescripción de una *Coolia* originalmente mal descrita).

Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N. & Zapata, M. 2011. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean) *Harmful Algae* 11:10-22. (Detectadas dos especies pseudocrípticas diferentes con descripción de una nueva especie de *Gambierdiscus*).

Fraga, S., Rodríguez, F. 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. (Enviado a PROTIST). (Se describe una nueva especie de *Gambierdiscus* y se da la primera cita de otra para el Atlántico)

En la última parte de la memoria se abordan las diferencias ecológicas entre las microalgas planctónicas y la bentónicas entre las que se encuentran *Gambierdiscus*, *Ostreopsis* y *Coolia*.

Fraga, S., Rodríguez, F., Bravo, I., Zapata, M. & Marañón, E. 2012. Review of the Main Ecological Features Affecting Benthic Dinoflagellate Blooms. *Cryptogamie, Algologie* **33**:171-79.

CAPÍTULO 1

GÉNERO *GYMNODINIUM*

BACKGROUND OF GENUS *GYMNODINIUM*

Until recently, most of the species of unarmoured dinoflagellates were grouped in the order Gymnodiniales Apstein 1909, and within this order, one single genus, *Gymnodinium* Stein, had and still have many species that continuously are being removed from it, to be grouped into new genera based on genetic sequences of some genes, pigment composition and ultrastructural studies. The criteria reviewed by Kofoid and Swezy (1921) to define the most important genera of unarmoured dinoflagellates were followed more or less rigorously until very recently when most of unarmoured dinoflagellates were ascribed to few genera: *Gymnodinium* Stein, 1878, *Gyrodinium* Kofoid & Swezy, 1921, *Amphidinium* Claperède & Lachmann, 1859, *Katodinium* Fott, 1857, and *Cochlodinium* Schütt, 1886. Differences among genera were based on the position of the cingulum in relation to the apical - antapical axes of the cell and if it was displaced or not. If the cingulum was placed towards the apical end, then it was genus *Amphidinium*, but if it was posteriorly placed it was, *Katodinium*. If the cingulum was more or less centered in the longitudinal axis, they were, *Gymnodinium* or *Gyrodinium*. The difference among these last two genera was based on the cingulum displacement. If it was bigger than 1/5 of the total length of the cell, then it was a *Gyrodinium*, but if it was less than that, it was a *Gymnodinium*. In the case that the cells were twisted and then the cingulum has more than 1.5 turns around the cell it is *Cochlodinium*.

These criteria alone are now no longer considered valid as they are artificial and, based on molecular and ultrastructural studies, *Gymnodinium* and *Gyrodinium* were redescribed and three more genera were created, *Karenia*, *Karlodinium* and *Akashiwo* with species previously considered to be *Gymnodinium* or *Gyrodinium* (Daugbjerg *et al.*, 2000). The new criteria are based mainly on the shape of the apical groove (Takayama, 1985), ultrastructure, pigment composition and molecular phylogeny. Since the critical review of Daugberg et al., (2000) several new genera were added to this group: *Togula* Flø Jørgensen, Murray & Daugbjerg, 2003; *Takayama* de Salas, Bolch, Botes & Hallegraeff 2003, (De Salas *et al.*, 2003); *Apicoporus* Leander & Hoppenrath 2008 (Sparmann *et al.*, 2008); *Barrufeta*, N. Sampedro & S. Fraga, 2011 (Sampedro *et al.*, 2011); *Moestrupia* G. Hansen & N. Daugbjerg, 2011 (Hansen & Daugbjerg, 2011); *Ankistrodinium* Hoppenrath, Murray, Sparmann & Leander 2012 (Hoppenrath *et al.*, 2012); *Testudodinium* Horiguchi, Tamura, Katsumata & A. Yamaguchi 2012, (Horiguchi *et al.*, 2012); *Paragymnodinium* Kang, Jeong, Moestrup, & Shin; *Bispinodinium* N. Yamada et Horiguchi, 2013 (Yamada *et al.*, 2013).

Based on ribosomal DNA sequences, Gymnodiniales are polyphyletic and some groups are very distant in the gene trees. Even in the clade known as *Gymnodinium* s.s.

some species are included in different genera showing that a profound revision of naked dinoflagellates is needed (Reñé et al 2013). *Gymnodinium catenatum* Graham is one of the species included in the *Gymnodinium* s.s. although it is probable that in the future it will be transferred to a new genus including other species as *Gymnodinium nolleri* M. Ellegaard & Moestrup (Ellegaard & Moestrup, 1999), *Gymnodinium microreticulatum* C.J.S. Bolch, Negri & G.M. Hallegraeff (Bolch *et al.*, 1999), *Gymnodinium trapeziforme* Attaran-Fariman & Bolch (Attaran-Fariman *et al.*, 2007) and *Gymnodinium inusitatum* H. Gu (Gu *et al.*, 2013). These species form a monoplyletic group and share some morphological characteristics like the formation of microreticulate cysts, reflecting amphiesmal vesicles in the motile cells.

GYMNODINIUM CATENATUM GRAHAM

In March 1939, the presence of enormous numbers of the dinoflagellate *Alexandrium catenella* (Whedon y Kofoid) Balech, or «some species resembling it» were reported in the inner part of the Gulf of California (Gilbert & Allen, 1943). Formalin fixed samples of this bloom were supplied to Herbert W. Graham by Winfred E. Allen and the chain forming unarmored dinoflagellate *Gymnodinium catenatum* Graham (Graham, 1943) was described as a species new to science (Graham, 1943) using the criteria valid at that time (Kofoid & Swezy, 1921).

The description was based on formalin fixed samples, and for this reason the author already reported that some distortion had taken place as it was evidenced by a considerable variation on the body contours. Nevertheless he decided that, despite this problem, worthwhile a description since the body shape and chain formation were so characteristic that the species can be easily recognized in preserved samples once an acquaintance with it is made (Graham, 1943).

Gymnodinium catenatum (Fig. 1) was described by Graham as:

> *Gymnodinium catenatum sp. nov.*
>
> *Dimensions. Length, 30 (22-33) μ; transdiameter, 36 (30-46) μ*
>
> *Description. Body circular to squarish in ventral view, truncate posteriorly with deep notch at sulcus, rounded anteriorly in anterior members of chains. The epicone of all other members of chains pointed where there is attachment to the sulcus of the cell ahead. In anterior view the body is subcircular. The species seems to occur in two forms. In one the length is approximately equal to the transdiameter; in the other there is considerable dorsoventral flattening so that the length is only about 0.64 times the transdiameter. Girdle median, without displacement,*

wide, about 0.6 transdiameter, deeply impressed. Sulcus extends from near apex to antapex. Cytoplasm greenish-yellow, packed with irregularly rounded refractory bodies and food vacuoles. Ectoplasm forma a thin pellicle in some stages. No striae or other surface markings observed. The protoplast has an unusual capacity to maintain its shape in formaldehyde preservative. This ability could not be ascribed to the presence of a firm pellicle since in many specimens it was impossible to demonstrate any semblance of pellicular structure. The nucleus is centrally located, moniliform, large, thick and slightly lunate. The species occurs characteristically in chains of many individuals; as many as 29 units have been counted in a single chain. Many of the single individuals in preserved samples may represent broken chains. The cytoplasm is continuous through the chain. The apex of one individual is drawn out into a narrow extension which joins the posterior part of the sulcus of the next member. (Fig. 1)

It was not until more than twenty years later that this species was observed again, in 1959 in Hiroshima Bay (Fig. 2) (Hada, 1967) and two years later in Mar del Plata (Fig. 3) (Balech, 1964, Balech, 1988). In these cases, living cells, and not only fixed, were studied and then some differences with the original description were reported. In both publications the presence of chloroplasts that were not observed in the original description were reported, and Balech (1964) also reported a sulcal displacement that in one of his figures is just 1/5 of the total length of the cell, the limit to differentiate genus *Gymnodinium*

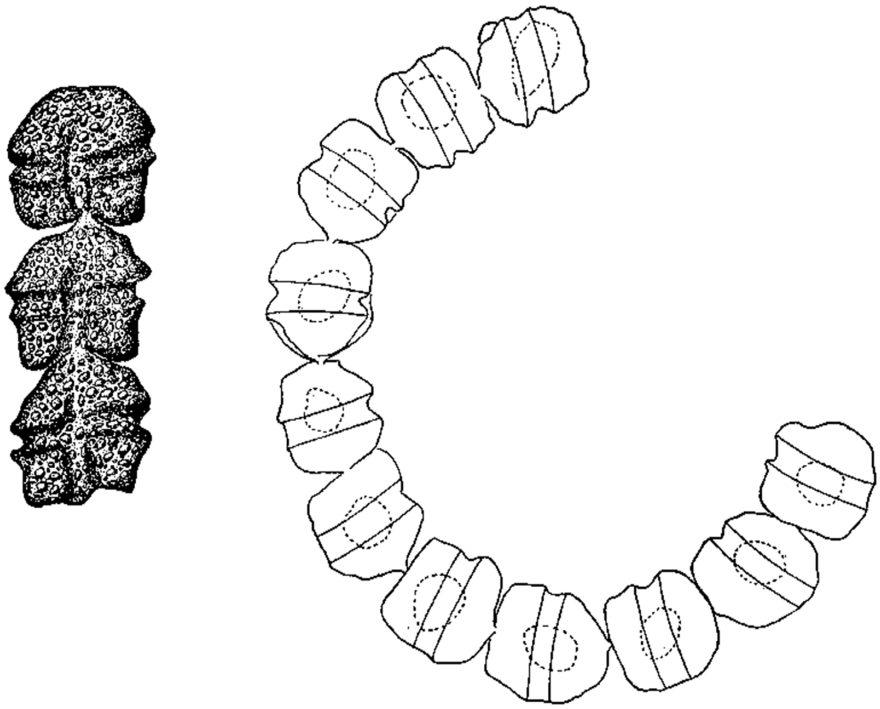


Figure 1. Original description of *Gymnodinium catenatum* after Graham (1943).

Figure 2. *Gymnodinium catenatum* after Hada (1967).

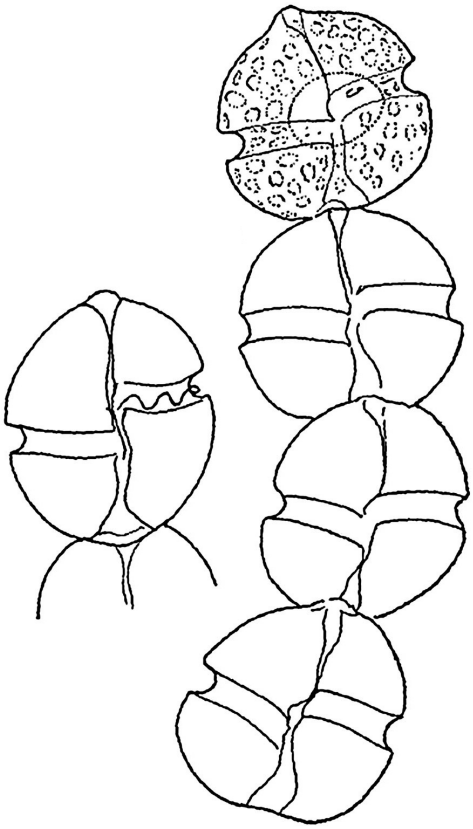


Figure 3. *Gymnodinium catenatum* after Balech (1964).

from *Gyrodinium* according to the criteria of Kofoid and Swezy in use at that time (Kofoid & Swezy, 1921).

GYMNODINIUM CATENATUM IN GALICIA

The following time that *G. catenatum* was observed in the world was in Galicia in October 1976 when a PSP outbreak caused by blue mussels was recorded (Gestal *et al.*, 1980, Lüthy, 1979). Just after the first cases were diagnosed, on October 26th, the local health authorities took four surface samples in the bay of Bueu, Ría de Pontevedra. In most of the samples, diatoms were more abundant than dinoflagellates suggesting that the hydrographic conditions had already changed since the day that the dinoflagellate bloom caused the PSP event (Estrada, 1976). In those samples, a chain forming

dinoflagellate was the most abundant dinoflagellate species with relatively high abundances from 6,000 to 22,000 cells'»L^{-1}, that together with the fact that the samples were taken just after the PSP outbreak, make it the most suspicious species of being the causative agent, although a few cells of *Gonyaulax* of the *tamarensis* group (=*Alexandrium* sp.) were also observed in the samples. At that moment, it was not possible to properly identify it.

Few years after the 1976 Spanish outbreak, in April 1979, three children died after eating bivalves at the time a conspicuous bloom of *G. catenatum* in the bay of Mazatlán, México, near the South of the Gulf of California (Mee *et al.*, 1986, Morey-Gaines, 1982) being this the first clear association of *G. catenatum* with PSP.

At the beginning of October, 1981, *G. catenatum* was observed again in Galicia (Estrada *et al.*, 1984b), first in Rías de Pontevedra and Arousa and later in Ría de Muros and Ría de Vigo. By October 15th a conspicuous «red tide» formed by *Prorocentrum triestinum* with concentrations of more than eight millions cells'»L^{-1} and *Prorocentrum rostratum*, with 186.000 cells'»L^{-1} was observed in the outer part of Ría de Vigo, in which *G. catenatum* was also present, but at concentrations lower than 10,000 cells'»L^{-1}. It is interesting to quote that these two *Prorocentrum* species, that were not previously recorded in Galicia, had been observed off the coast of Sines at the South of Portugal together with *G. catenatum* one month before (Estrada, pers. com.).

From here, *G. catenatum* became a non rare species in Galicia and it was observed again almost every year, and sometimes in bloom concentrations that make bivalve toxic for long periods.

In 1985 several strains of *G. catenatum* were isolated andd brought into laboratory culture providing unlimited material for study.

Description based on LM of field and cultured living cells.

Dinoflagellate 31-39 µm long and 37-42 µm wide. It forms chains usually of 2n cells (n=1, 2, 3, 4). In field samples, chains of more than 40 cells were occasionally observed being the chains of 4 and 8 cells the most common. In exponentially growing cultures long chains of more than 100 cells were observed. The normal chains were more or less straight and swam with undulations when they were healthy, but they became curved and helicoidal when they were weak or death (Figs. 4,5). The length/width ratio was variable but most of the times they were wider than long. Cells were slightly dorsoventrally flattened and kidney shaped in apical view. The epicone is slightly smaller than the hypocone and has a concave silhouette (Fig. 6). In ventral view, the apex of the anterior cell of the chains were rounded while in the other cells it had an elongation that

Figure 4. *Gymnodinium catenatum*. LM image of part of a chain of living cultured cells. Scale bar: 20 µm.

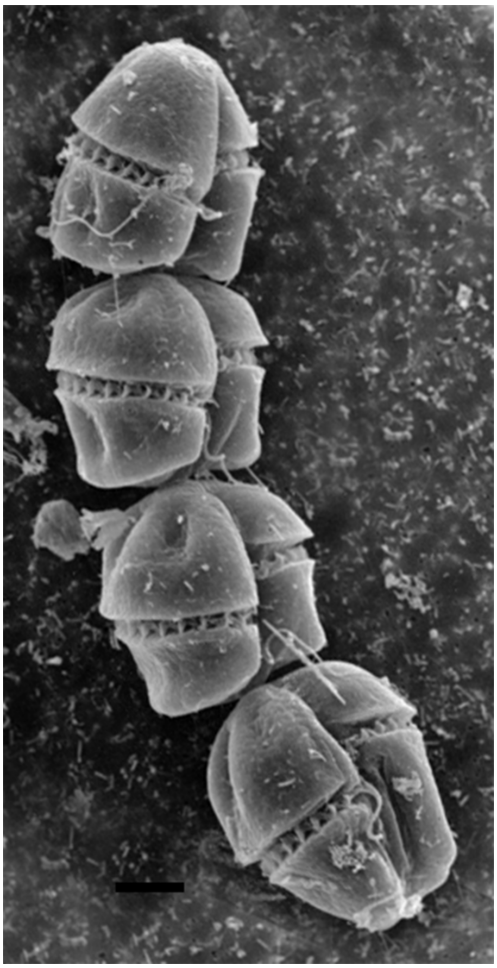


Figure 5. *Gymnodinium catenatum*. SEM image of a four celled chain of cultured cells. Scale bar: 10 µm.

connected with the antapex of the anterior cell in the chain although this connection was not visible when the cells were very close together. The hypocone in all the cells except the last one, was truncated and even concave with irregular margins, while in the last cells was rounded and convex. Longitudinal sulcus was narrow and deep penetrating in the epicone. The cingulum was wide and deep, having a displacement that could be higher that 1/5 of the total cell length. The nucleus was oval and central. In field samples some refringent round bodies that became dark when when fixed with Lugol's solution were very common. Chloroplasts were small and numerous. When the cells started to be damaged under the microscope heat, they shed a pellicle and the cells became more

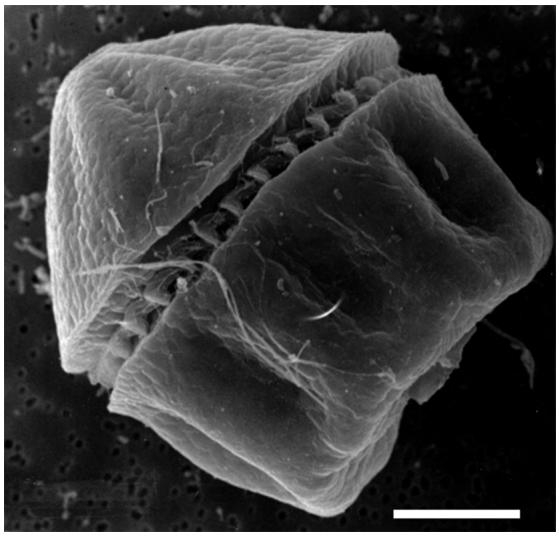


Figure 6. *Gymnodinium catenatum*. SEM image of the dorsal view of a cultured cell. Scale bar: 20 µm.

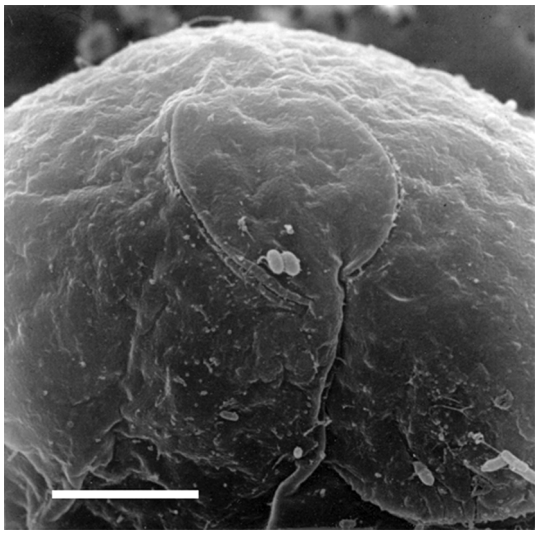


Figure 7. *Gymnodinium catenatum*. SEM image of the acrobase of a cultured cell. Scale bar: 20 µm.

rounded and the edges of sulcus and cingulum were less pronounced. The longitudinal flagellum had approximately the same length than the body of the cell.

In observations based on SEM of cultured cells, the amphiesmal vesicles are clearly seen in the cell membrane. At the apex of the cells a small horseshoe-shaped groove can be seen (Fig. 7) which is not directly connected to the sulcus prolongation on the epicone.

G. catenatum was studied in samples from Mazatlán (Gulf of California) by Morey-Gaines (Morey-Gaines, 1982). However, there are certain differences between the organisms described as *G. catenatum*. According to Graham, who observed samples fixed in formaldehyde, they have no cingular displacement, in contrast to what found by other authors. In addition, the cells observed by Graham appear relatively wider than others. However, as the original description was made based on fixed material, it is doubtful the importance attributed to these differences. Graham did not indicate the presence of chloroplasts, which could be due, as suggested by Morey-Gaines (1982) to the bleaching effect of formaldehyde. In addition, certain characters described by Balech, on live cells, are different from those observed in specimens of Galician rias, the contour of the cells from Mar del Plata is elliptical, while that of those from the Galician Rias is more quadrangular, the longitudinal sulcus extends to the apex and widens at the base of the Argentinean cells, while in the rías the sulcus penetrates into the epicone and is not

expanded at the base. The horseshoe apical groove seem to correspond to the «acrobase» described by Chatton and Hovasse (1934).

In most of the cases that *G. catenatum* was observed, it was in high concentrations, even forming «red tides». In the Gulf of California, Graham (1943) quotes concentrations of until 100.000 cells/L and when it was associated with toxicity in Mexico was forming a «red tide» (Mee et al., 1986, Morey-Gaines, 1982).

After the observations of the chains of small cells at the time of a bloom of *G. catenatum* in Galicia in 1981, a strain of a chain forming dinoflagellate resembling *G. catenatum* was isolated in Ría de Vigo (Bravo 1986). The same species bloomed in coastal waters of Valencia, West Mediterranean sea in 1988. At a first look, it was thought to be the toxic *Gymnodinium catenatum*, but after a more carefully observation the cells showed notorious differences. New strains were brought into culture and they were non toxic.

Ccmpared with strains of *G. catenatum*, the cells of the new strains were smaller in size, slightly different in shape, formed shorter chains, had bigger and more rounded chloroplasts and had a different behavior. These data lead to the description of a new species, *Gyrodinium impudicum* Fraga & Bravo (Fraga *et al.*, 1995b).

GYRODINIUM IMPUDICUM SP. NOV. (DINOPHYCEAE), A NON TOXIC, CHAIN-FORMING, RED TIDE DINOFLAGELLATE

NOTE: After the publication of this paper, Daugbjerg et al (2000) tranferred *Gyrodinium impudicum* to genus *Gymnodinium* as *G. impudicum* (S. Fraga & I. Bravo) G. Hansen & Ø. Moestrup.

ABSTRACT

A new dinoflagellate *Gyrodinium impudicum* sp. nov. is described from Valencia Harbour, Ría de Vigo (Spain) and Fusaro lagoon (Italy). It is a Gymnodiniaceae with cingulum a descending left spiral, displaced between 1/3 and 1/4 of the total length of the cell and sulcus without torsion, reasons why it is assigned to *Gyrodinium*. This chain-forming red tide organism has been misidentified in several previous papers as *Gymnodinium catenatum* Graham or as *Polykrikos schwartzi* Bütchli. It is negative for paralytic shellfish poisons, however has different cell shape and acrobase being smaller in size. It has caused blooms in several areas but no associated harmful effects have been reported.

INTRODUCTION

Massive blooms of an unarmoured chain-forming dinoflagellate resembling the toxic *Gymnodinium catenatum* Graham (1943), have been observed on the Mediterranean coast of Spain off Valencia since 1988 (Alcober, personal communication) and on the Catalan Coast in 1993. The cells are usually embedded in large amounts of mucus, and the blooms did not cause any known damage to fish or shellfish in the areas in which they were observed.

G. catenatum is a toxic chain-forming dinoflagellate that causes shellfish poisoning at its type locality, the Gulf of California (Mee *et al.*, 1986) as well as in the coastal waters of other parts of the world e.g. the Iberian Peninsula (Anderson *et al.*, 1989, Franca & Almeida, 1989), Tasmania (Oshima *et al.*, 1987) and Japan (Ikeda *et al.*, 1989).

Observations of a 'small *G. catenatum*' (Estrada *et al.*, 1984a, Anderson et al., 1989), and the isolation by us of new non toxic strains of a dinoflagellate resembling *G. catenatum*, led us to investigate those cases, and to identify the new species.

MATERIAL AND METHODS

Cultures and field samples

Several different strains of *Gyrodinium impudicum* sp. nov. were isolated: a) Strains GY1VA and GY2VA were obtained by pipetting vegetative cells from water samples taken in Valencia Harbour (Mediterranean coast of Spain) during a bloom in August 1992, b) strain GY5V is from vegetative cells from Ría de Vigo (NW coast of Spain) in July 1992, and c) strains GY3VA and GY4VA are from sediment (probably from resting cysts) taken in December 1991 off Valencia and incubated in July 1992. Several chains were picked up with a pipette and cultured in polystyrene well plates in enriched K medium (Keller & Guillard, 1985) at a salinity of about 34, 17-18°C and a 14:10 h L:D cycle. Samples of mud were sonicated, and sieved, and the sediment fraction between 20 ìm and 75 ìm was incubated at the above conditions. When chains of cells were observed in a well, they were isolated by a micropipette and cultured under the same conditions. A culture of strain 10B isolated in Fusaro Lagoon (Italy) and kindly provided by Dr Marina Montresor was maintained under the same culture conditions. Two strains of *Gymnodinium catenatum* (GC19V and GC7B) from Ría de Vigo were used for comparative studies. All the strains used in this work are kept in the Culture Collection of Marine Phytoplankton of the IEO in Vigo.

Field samples of *G. impudicum* were taken from the surface with a bucket during a bloom in August 1992 in Valencia Harbour.

Light microscopy

Living samples were examined under bright field, Nomarski and epifluorescence using a High Pressure Mercury lamp and filter-set Zeiss 487709. Some cultured specimens were fixed for 2 h with OsO_4 at a final concentration of 1.5 % in the refrigerator and observed by light microscopy. Field samples from Valencia were fixed in Lugol's solution.

Electron microscopy

Five mL of culture were fixed at room temperature for 2 h with a mixture of 1.2 mL of 5% OsO_4 and 0.25 mL of 30% glutaraldehyde prepared just before fixation. They were filtered through a 13 mm diameter and 12 ìm pore size Nuclepore membrane filter to obtain cells without mucilage. Samples were dehydrated by serial dilutions of ethanol, critical point dried with CO_2, and examined under a Hitachi S-570 Scanning Electron Microscope. The strains examined under SEM were GY1VA (*G. impudicum)* and GC7B (*G. catenatum).*

Toxins

The potential presence of PSP toxins was checked by the standard AOAC mouse bioassay (Williams, 1984) on extracts from cultures and from field samples from the bloom event in Valencia. HPLC analyses follow the method of Oshima *et al*. (1989), as modified by Franco & Fernández-Vila (1993).

Pigments

25 mL of culture were filtered onto Whatman GFF glass fiber filters under low vacuum and inmediatly frozen. Pigments were extracted by sonication in 95% methanol and HPLC separation was carried out with a Beckman System Gold which includes a 126 solvent module and a 168 UV-Vis diode array spectrophotometer interfaced with a Merck-Hitachi F1050 spectrofluorimeter (excitation wavelength 440 nm emission 660 nm); 200 ìL of the extract were injected into a reversed-phase column Lichrospher PAH (E. Merck), 250 x 4.6 mm i.d. (polymeric octadecylsilica, 5 ìm particle size).

The HPLC method used has been previously described (Garrido & Zapata, 1993) and was applied with slight modifications: a linear gradient from 80% A (methanol : 1M ammonium acetate 8:2 v/v) to 100% B (acetone) was pumped for 23 min. Subsequently, an isocratic hold at 100% B was used for 5 min at a flow rate was 1.2 mL min^{-1}. The column was thermostated at 17°C by means of a water bath in order to increase the resolution between peridinin and chlorophyll c pigments. Pigment identification was obtained by diode array spectroscopy in eluent, and it was confirmed by absorption spectrum in standard solvents (acetone and diethyl ether).

Sterols

(Hallegraeff *et al.*, 1991) observed that sterol composition changes little with the age of the culture, and samples were therefore taken only during the exponential phase. Cells were concentrated by centrifugation and extraction followed the method of Bligh & Dyer (1959). Cell pellets were homogenized by sonication in 10 mL of $CHCl_2$:MeOH (1:2) followed by two extractions in 10 mL $CHCl_2$. Apolar fractions were rinsed with 50 mL of distilled water. The chloroform fraction was dried, and the residue was saponified by a KOH (1N) : MeOH (1:5) reflux for 3 h. The unsaponifiable fraction was extracted and split into three fractions of 15 mL of hexane : ethilic ether (9:1).

Sterols from the unsaponifiable fraction were converted into trimethylsilyl ethers (OTMSi-ethers) after treatment with bis(trimethylsilyl)trifluoracetamide (BSTFA). The derivates were analyzed by Gas chromatography (GC) in a Perkin-Elmer Autosystem chromatograph equipped with a 60m x 0.32mm i.d. and 0.25 ìm thick TRB 1 linked

phase column and detected by a flame ionization detector (FID). Working conditions were: injector temperature of 310°C with the split closed during the first minute. Helium was used as carrier gas at 35 psi and a split relation of 1:50. The oven temperature was 50°C for 1 min, and it was increased to 150°C at a rate of 30°C min^{-1}, to 250°C at a rate of 2°C min^{-1}, and to 300°C at a rate of 7°C min^{-1}, maintaining this temperature for 30 min. The FID temperature was 310°C. A cholesterol standard was used to test the analytical system.

OBSERVATIONS

Gyrodinium impudicum *Fraga and Bravo sp. nov.*

Figs 7 - 16

Diagnosis*: Cellulae 14-37 µm longitudine et 16-32 µm latitudine. Typicas quattuor cellularum catenas formant, quamquam catenae breviores vel etiam cellulae solae observari possunt. Nucleus centralis, quamquam leviter emotus ad hypoconum in cellula antica catenae et ad hypoconum in postica. Cingulum profunde excavatum, emotum inter 1/3 et 1/4 a tota cellulae longitudine. Sulcus angustus, penetrans in epiconum usque ad apicen, eum circumdans per dorsualem partem de sinistra ad dexteram ad profundam acrobasem formandam.Figura 1 ostendunt holotypum segregatum ab aquae exemplo capto in Valentiae porto.*

Cells 14-37 µm long and 16-32 µm wide. Typically, forming chains of 4 cells, although longer and shorter chains or solitary cells can be observed. The nucleus is central, but slightly displaced towards the hypocone in the anterior cell of a chain, and towards the epicone in the posterior one. The cingulum is distinct with a displacement between 1/3 and 1/4 of the total length of the cell. The sulcus is narrow, penetrating into the epicone as far as the apex, where it turns anticlockwise viewed from the apex (Figs 3, 4), to form a deep acrobase.

Holotype: Fig. 8 from strain GY1VA isolated by I. Bravo from a water sample collected during a bloom in Valencia Harbour in August 1992.

Etymology: Latin *impudicum* lewd, referring to the phallus like appearance.

Type locality: Valencia Harbour (Spain)

Synonyms: *Gymnodinium catenatum* Graham (strain 3V) in Bravo (1986). *Gymnodinium catenatum* Graham in Carrada *et al.* (1991). *Gyrodinium* sp. in Fraga *et al.* (1995a).

Distribution: *G. impudicum* has been observed forming massive blooms in the coastal waters off Valencia (J. Alcober, personal communication), on the Catalan Coast of the

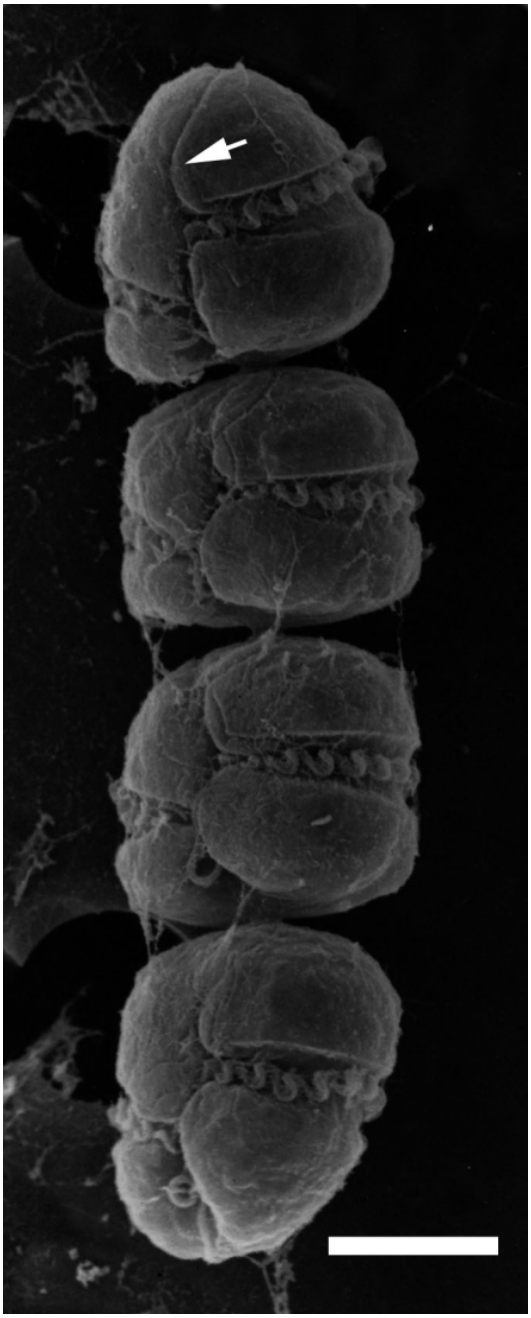


Fig. 8. SEM picture of *Gyrodinium impudicum* sp. nov.. Chain of four cells. Arrow marks connection of sulcal intrusion in the epicone with acrobase. Scale bar: 10 µm.

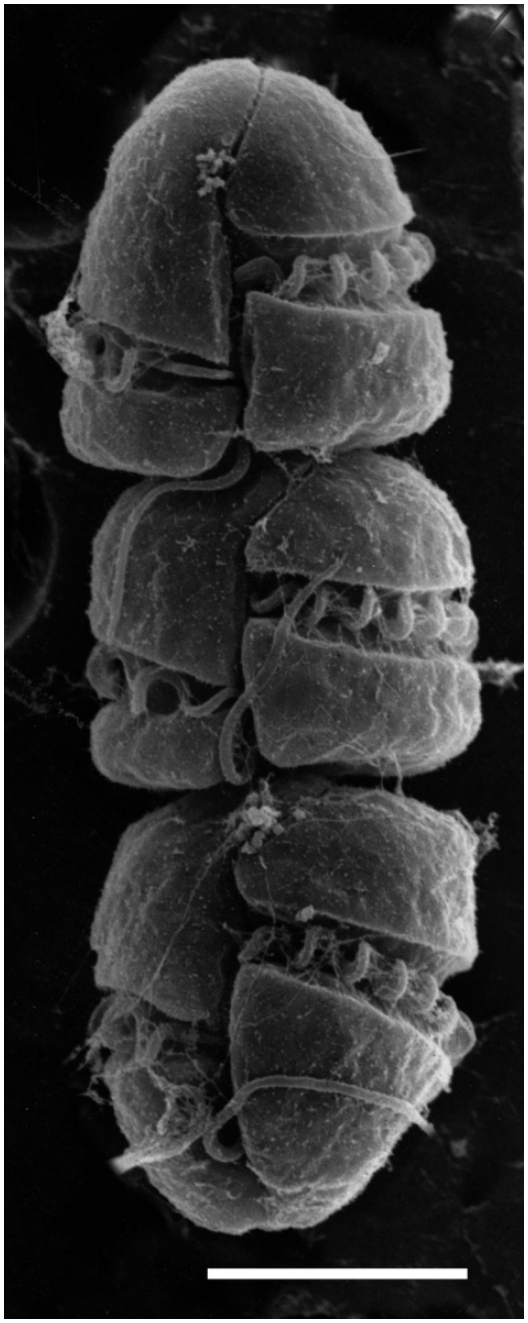


Fig. 9. SEM picture of *Gyrodinium impudicum* sp. nov.. Chain of three cells. Scl Scale bar: 10 µm.

Mediterranean Sea (this paper)and in Fusaro Lagoon (Italy) as *G. catenatum* (Carrada *et al.*, 1991, Carrada *et al.*, 1988). It has been observed, but not forming blooms, in Ría de Vigo (Spain) (This paper and Estrada (1984a)) as a small form of *G. catenatum*; Bravo (1986) as *G. catenatum*, and in Portuguese (M.A. Sampayo, personal communication) and Australian waters (D. Hill, personal communication).

Description

The cells are 14-37 µm long and 16-32 µm wide. Typically, they form chains of 4 cells (Fig 8, 13), both in field samples and in laboratory cultures, although longer (Figs 15, 18) and shorter chains (Fig. 16) or solitary cells (Fig. 17) can be observed. The size of the cells generally increases towards the posterior end of the chain (Figs 9, 14, 16). The cells have four different shapes according to their relative position in the chain, or when they appear as solitary cells. In a chain, the anterior cell has pointed epicone and flattened hypocone (Figs 9, 20), the central cells have flattened epicone and hypocone (Figs 9, 22), and the posterior cell of a chain has a flattened epicone and pointed hypocone (Fig. 9). When the cells are solitary, both epicone and hypocone are pointed (Fig. 17). The epicone is usually larger than the hypocone (Figs 14, 20). The nucleus is central, but slightly displaced towards the hypocone in the anterior cell of a chain, and towards the epicone in

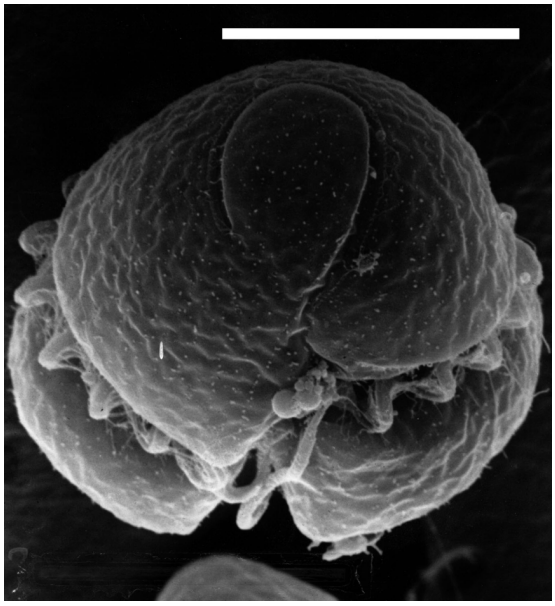


Fig. 10. SEM pictures of *Gyrodinium impudicum* sp. nov.. Oblique apical-ventral view of an anterior cell of a chain showing the acrobase. Scale bar: 10 µm.

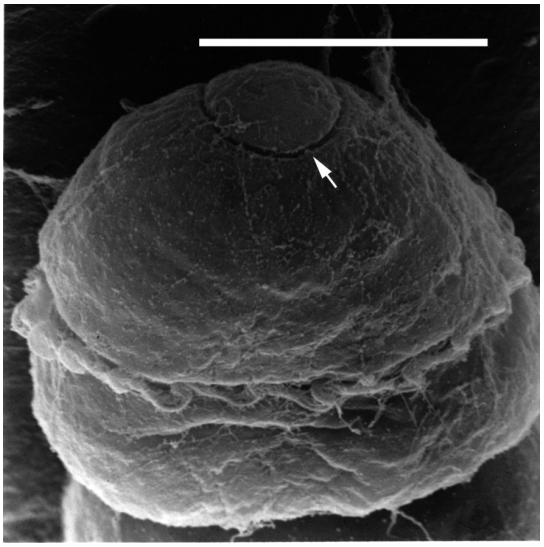


Fig. 11. SEM pictures of *Gyrodinium impudicum* sp. nov.. Oblique apical-dorsal view of an anterior cell of a chain showing the acrobase (arrowhead).Scale bar: 10 µm.

the posterior one. The cingulum is distinct with a displacement between 1/3 and 1/4 of the total length of the cell (Figs 9, 20, 23). The sulcus is narrow and longitudinal, penetrating into the epicone as far as the apex, where it turns anticlockwise viewed from the apex (Fig 3, 4), to form a deep acrobase, which can be observed in side view as an indentation (Figs 20, 21, 23) visible even with light microscope. No amphiesmal vesicles or striae have been observed in the cellular surface, but in living cells under Nomarski illumination it appears to be finely reticulated but this was not visible under SEM. It could be an optical artifact or it may have been lost when the cells were fixed. In SEM preparations, the surface of *G. impudicum* appears covered by small white dots. Two cells in a chain are interconnected in the dorsal side of the acrobase in the posterior cell (Fig. 12), and the antapical region of the anterior cell which appears concave (Figs 22, 23). Numerous small and elongated chloroplasts are present. In many chains it is possible to observe a red body that fluoresces white under blue excitation.

In culture, the behaviour of *G. impudicum* is very peculiar. Most of the time, the cells lie at the bottom of the culture vessel with a slow beat of the flagella as the only apparent sign of life. Sometimes the chains swim very fast in a straight line. They produce large amounts of mucus, which is easy to observe if the culture is contaminated by bacteria or small algae (Fig. 19). The mucus, which is also observed in material taken directly from in the sea, makes it difficult to obtain clean images under SEM, and to obtain concentrated samples for analysis of toxins, pigments, etc.

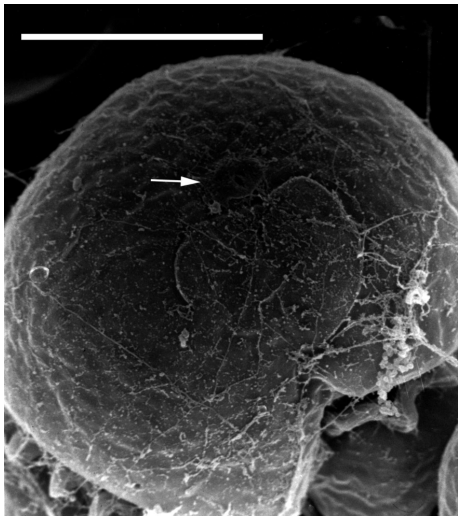
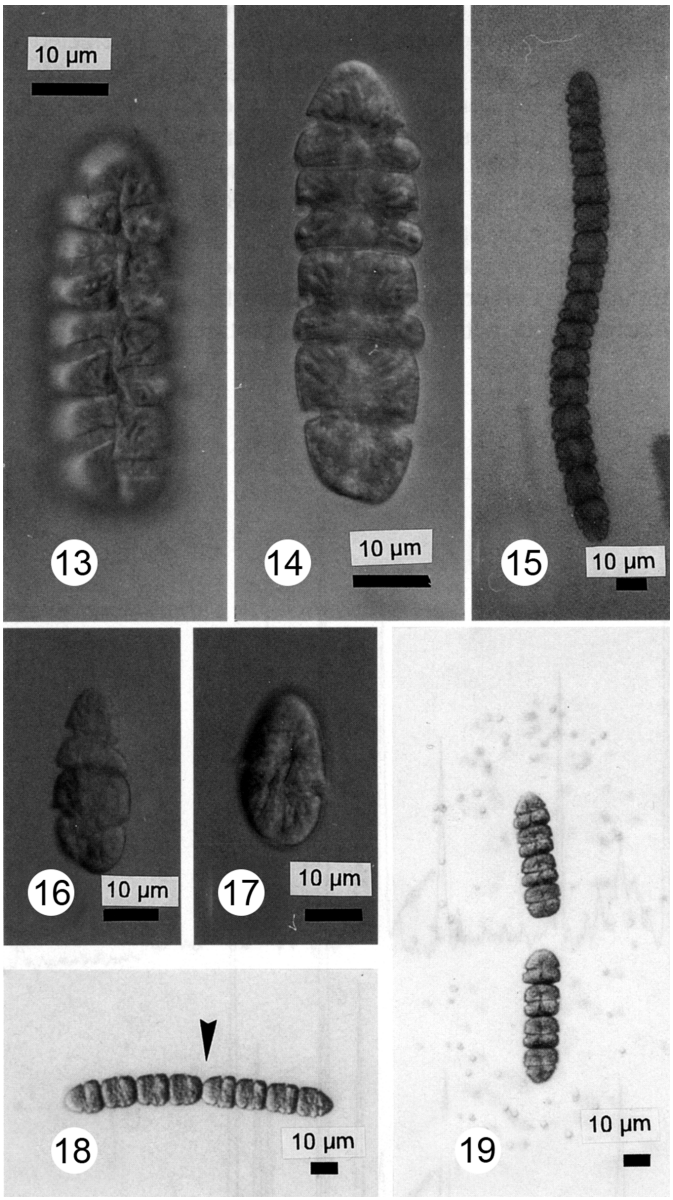


Fig. 12. SEM picture of *Gyrodinium impudicum* sp. nov.. Apical view of an intercalary cell of a chain showing acrobase and the point of connection with a neigbouring cell (Arrowhead).



Light micrographs of living cells of *G. impudicum* sp. nov.. Fig. 13. Ventral view of a chain of four cells showing cingular displacement (note that image is inverted). Fig. 14. Dorsal view of a chain of four cells in which epicones are clearly larger than hypocones. The size of the cells increases towards the end of the chain. Fig. 15. Exceptionally long chain of 16 cells. Fig. 16. Pair of cells in which the posterior cell is much larger than the anterior one. Fig. 17. Single cell showing both pointed epicone and hypocone. Fig. 18. Chain of eight cells at the beginning of division into two four-celled chains. Fig. 119. Two chains of four cells embedded in 'dirty' mucus soon after division from an eight-celled chain. All scale bars of 10 µm.

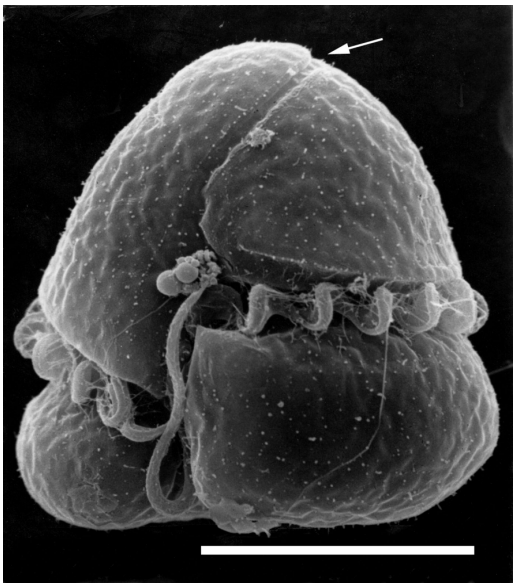


Fig. 20. SEM picture of *G. impudicum* sp. nov.Ventral view of a detached anterior cell of a chain showing acrobase (arrowhead) and cingular displacement. Scale bar: 10 µm.

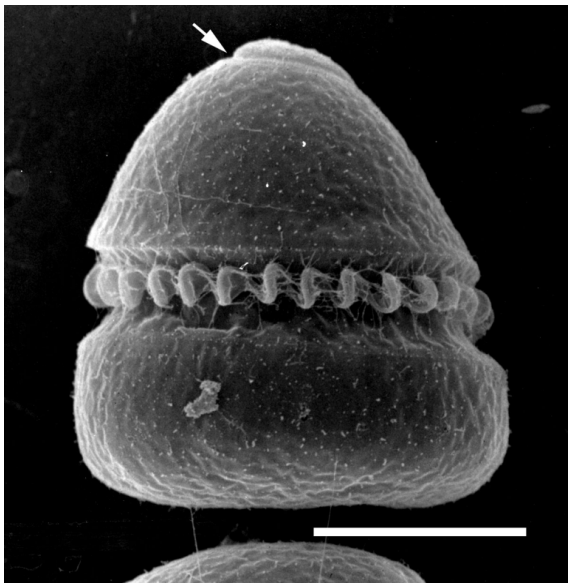


Fig. 21. SEM picture of *G. impudicum* sp. nov. Dorsal view of a detached anterior cell of a chain showing indentation of acrobase (arrowhead). Scale bar: 10 µm.

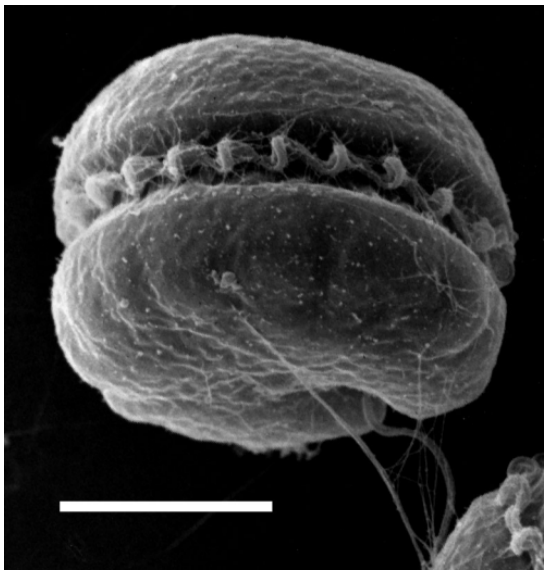


Fig. 22. SEM picture of *G. impudicum* sp. nov. Dorsal view of a detached intercalate cell of a chain showing both flattened epicone and hypocone. Scale bar: 10 ¼m.

Fig. 23. SEM picture of *G. impudicum* sp. nov. Lateral view of the two anterior cells of a chain showing cingular displacement, indentation of acrobase (arrowhead) and point of connection of two cells. Scale bar: 10 µm.

Comparisons with other reports

In field samples, Estrada *et al* (1984a) observed together with *G. catenatum* much smaller cells (17 ìm) in shorter chains. These cells were in all probability *G. impudicum* as the two species commonly occur simultaneously in the Galician rias, and both the size of the cells and the length of the chains correspond well with *G. impudicum*. Fraga & Sánchez (1985) reported on a culture of a dinoflagellate resembling *G. catenatum*. It was smaller in size and lacked the round refringent bodies observed in field samples that turn dark brown when fixed with Lugol (Estrada et al., 1984a). This culture (strain 3V) was described by Bravo (1986). It was smaller (20-35 ìm long, 16-26 ìm wide) than the typical *G. catenatum* and the chains shorter. Bravo's description and illustrations (fig 1-4) correspond well with the characteristics of *G. impudicum*. The strain was later analyzed by HPLC (Anderson et al., 1989) and shown to be non-toxic, and it is now deposited in the Provasoli-Guillard Center for Culture of Marine Phytoplankton under the designation CCMP413. The cells referred to *G. catenatum* from Fusaro Lagoon (Tyrrhenian Sea) by Carrada *et al*. (1991, , 1988) is *G. impudicum*, according to the morphology shown in their figs 5a and 5c, as well as other details such as size and typical number of cells per chain. We have observed a new culture (strain 10B from Stazione Zoologica di Napoli) obtained from Fusaro Lagoon, and identified it as *G. impudicum*, confirming that the report of Carrada *et al*. (1991, , 1988) is *G. impudicum.* There are some reports of other species that could be attributable to *G. impudicum*: A Japanese culture isolated from a red tide on the coast of Fukuyama, Hiroshima Prefecture (Japan) was reported by Iwasaki (1971) as *Polykrikos schwartzi*. Ishio *et al.* (1977) studied the same culture as Iwasaki (1971) and reported it as *impudicum*. It is possible, as well, that some reports of red tides of chain-forming *Cochlodinium* were in fact *G. impudicum*.

Biochemistry

No toxins of the saxitoxin group were detected by HPLC analysis of field samples taken off Valencia and Catalunya during conspicuous blooms of this species, nor from laboratory cultures. Mice bioassays of cultures of *G. impudicum* were also negative.

The pigment pattern otained for *G. impudicum* (Fig. 24) shows the presence of chlorophyll c_2 and chlorophyll a (Chl a) as the main fluorescence compounds. No chlorophyll c_1 was detected and only allomeric and epimeric forms of Chl a appear as minor peaks. The carotenoids ($ë_{max}$nm acetone) peridinol (465), peridinin (472), dinoxanthin (417,440 and 470), diadinoxanthin (426, 448 and 479), diadinochrome (406,

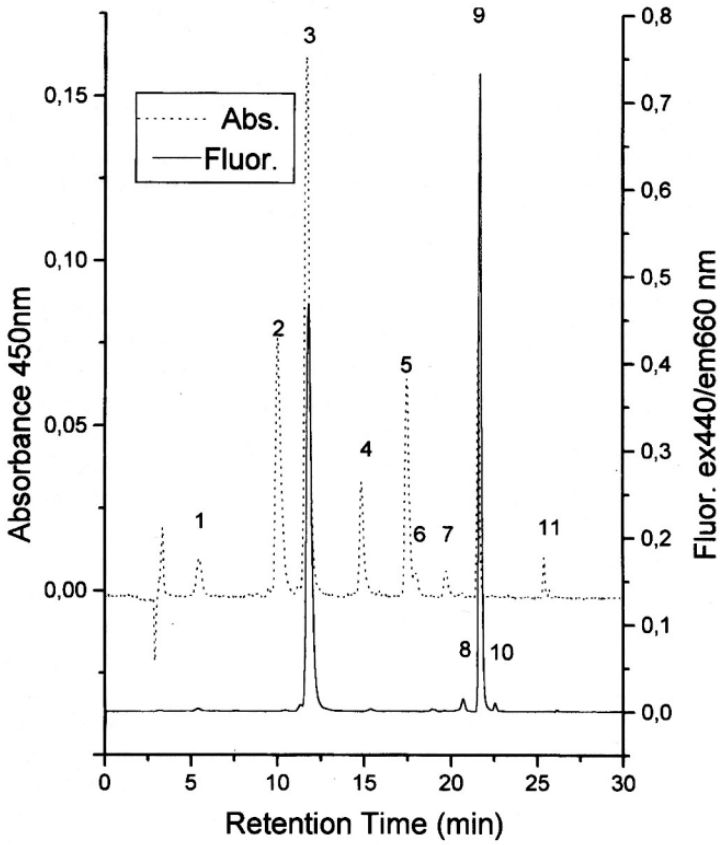


Fig. 24. HPLC chromatogram of pigments from *G. impudicum* sp. nov.. Upper trace absorbance at 450 nm, lower trace fluorescence chromatogram. Peak identification: 1, peridiniol; 2, peridinin; 3, chlorophyll c_2; 4, dinoxanthin; 6, diadinochrome; 7, diatoxanthin; 8, chlorophyll a allomer; 9, chlorophyll a; 10, chlorophyll a'; 11, ß-ß carotene.

429 and451), diatoxanthin (432, 453 and 480) and ß,ß-carotene (430, 451 and 478) were identified.

Figure 25 shows parts of gas chromatograms of sterols, as trimethylsiliyl ethers, of *G. impudicum* from Spain and Italy compared with *G. catenatum* cultured and analyzed at the same conditions. About 15 peaks can be observed, 13 of which having a higher retention time than that of cholesterol (64.5 min). The masses of the OTMSi derivatives measured by GC-MS ranged from 472 to 500, the same range reported by (Hallegraeff et al., 1991). Similar peaks, although with different relative concentrations, were found in the two strains of *G. impudicum* and in the two strains of *G. catenatum.* Nevertheless, there are some differences between the two species. In Fig. 17, peaks marked with an arrow in the chromatograms of *G. catenatum* (GC19V) are absent in *G. impudicum* (10B and GY5V). Similarly, one peak marked with arrows in *G. impudicum* were not observed in *G. catenatum*.

Ecology and behaviour

G. impudicum has been observed forming blooms at high temperatures and salinities: 22-24°C, 37.7 PSU, on the Catalan Coast (this paper); temperatures about 25-28°C in Valencia (Alcober, personal communication); and salinities ranging between 36 and 38 PSU in Italy (Carrada et al., 1991). In the later case, the temperature was not reported, but it was probably high. The bloom was in a coastal lagoon where evaporation is higher that freshwater inputs.

The places where *G. impudicum* blooms were observed, are generally polluted: our observations of a red tide in the harbour of Valencia were in an area affected by sewage. Fusaro Lagoon is heavily polluted by sewage (Carrada et al., 1991). Nevertheless, in Ría de Vigo, an area where nutrient inputs are mainly from upwelling and recycling, but not from sewage (Prego, 1992) and temperature and salinity rarely exceed 20°C and 35,7 PSU respectively, *G. impudicum* was observed and isolated into culture but has never been reported forming a bloom.

DISCUSSION

By light microscopy, this species is very similar to the toxic *G. catenatum* from which it is often difficult to distinguish, especially in Lugol fixed samples from the field. As the original description of *G. catenatum* is very poor, we compare in this paper *G. impudicum* with *G. catenatum*, based not only on the original description, but also on our cultures of *G. catenatum*, and the observations of Balech (1964), Morey-Gaynes (1982), Estrada *et al*. (1984a), Bravo (1986), Anderson *et al*. (1988), Rees & Hallegraeff (1991)

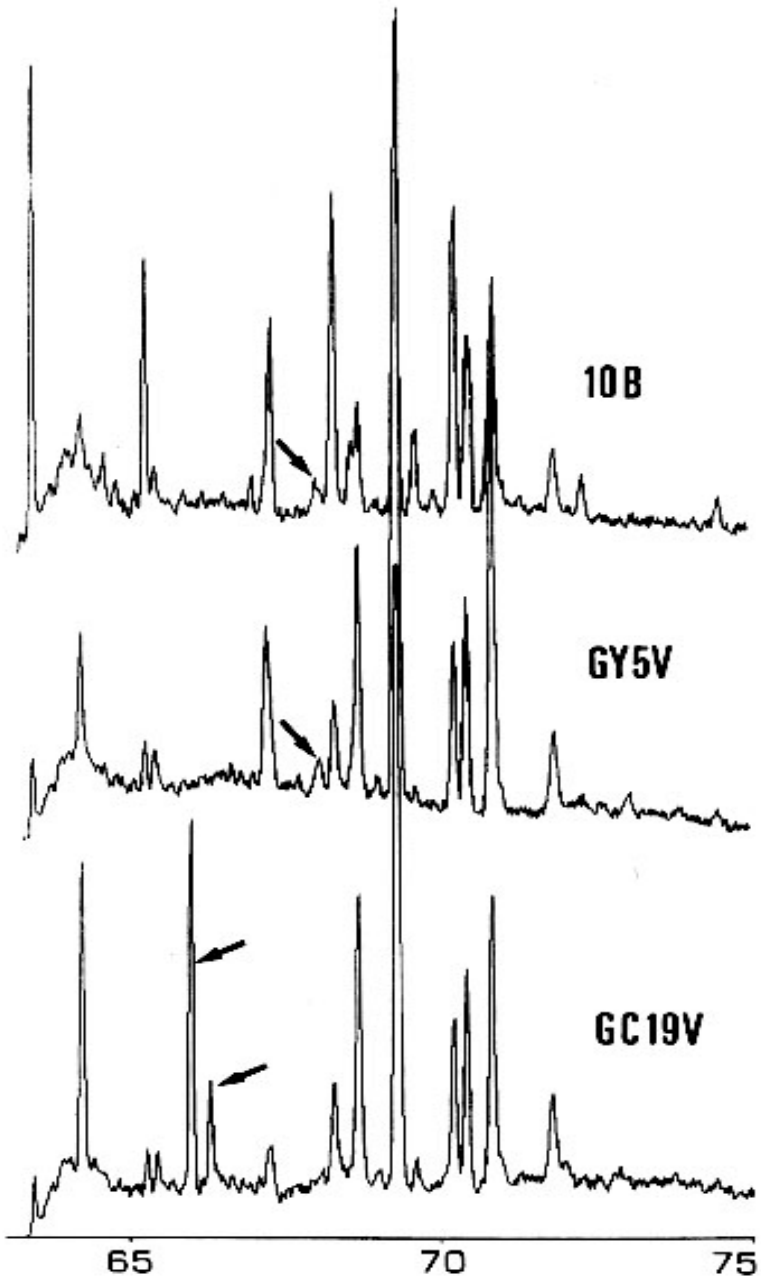


Fig. 25. GC chromatograms of sterols, as trimethylsilyl ethers, of *G. impudicum* sp. nov. (strains 10B and GY5V) and *G. catenatum* (strain GC19V). In the two upper chromatograms, arrows mark sterols of *G. impudicum* not observed in *G. catenatum*. In the lower chromatogram, arrows mark

and Ellegaard *et al.*(1993). The main differences between these two species (Table 1) have been summarized by Fraga *et al*. (1995a) where *G. impudicum* is called *Gyrodinium* sp.

G. impudicum is generally smaller in size than *G. catenatum* although the largest *G. impudicum* cells may overlap the smallest *G. catenatum* in size. The shape of the cells of the two species is also slightly different: In *G. impudicum* the hypocone is generally slightly smaller than the epicone, and the cell is always convex in side view, while the sides of the hypocone in *G. catenatum* are concave in healthy cells. The edges of the cingulum in *G. catenatum* are sharp and form a thick wing, while they are smooth in *G. impudicum*. The chains of *G. impudicum* mainly comprise four cells, and chains of 8 or 16 cells are very scarce and seen only during exponential growth (Figs 15, 18); According to our observations *G. catenatum* usually forms longer chains. In culture, it is very common to observe that soon after cell division in a four-cell chain producing an eight-cell chain, the fourth and fifth cells in the chain begin to acquire a pointed hypocone and epicone respectively, and the chain breaks into two new four-celled chains. (Figs. 18, 19) In a chain of *G. impudicum* the cells are closely attached, while in *G. catenatum* there is frequently an elongated connection. During cell division in *G. impudicum,* the cingula of the daughter cells are almost parallel, and a zig-zag pattern is not as apparent as in *G. catenatum*. Cingular displacement in *G. impudicum* is higher (1/3 to 1/4 of the cell length), which caused us to place this species in *Gyrodinium* following Kofoid & Swezy (1921). According to the original description (based on Formalin fixed material) *G. catenatum* has no cingular displacement. Subsequent descriptions reported a slight cingular displacement (Balech, 1964, Morey-Gaines, 1982, Anderson et al., 1988, Rees & Hallegraeff, 1991). Further research may result in the transfer of this species to another genus. The acrobase is different in these two species: in *G. impudicum* it is a prolongation of the sulcus from which it is not distinguishable (Figs 8-10, 20), while in *G. catenatum* it is not connected with the intrusion of the sulcus onto the epicone, and they are clearly two different structures. Ellegaard *et al.* (1993) reported a strain of *G. catenatum* from Denmark having a similar size as *G. impudicum* but the shape of the acrobase is very different from *G. impudicum*. When the cells of *G. catenatum* are under stress, they shed a membrane reflecting the shape of the cell (Estrada et al., 1984a), while this has not been observed in *G. impudicum*. The amphiesmal vesicles that are easily observed by SEM in *G. catenatum* have not been seen in *G. impudicum* using the same fixation.

The chloroplasts of *G. impudicum* are larger and more rounded than in *G. catenatum* which has more elongated chloroplasts (Rees & Hallegraeff, 1991).

Genetically *G. impudicum* is very different from *G. catenatum*, this being the definitive proof that they are different species (Zardoya *et al.*, 1995).

While no toxins were detected in *G. impudicum*, PSP toxins were detected in cultures of *G. catenatum* grown in the same culture medium and under the same conditions of temperature and light. Carrada *et al*. (1991) reported that cultures of the catenate species from Fusaro Lagoon were toxic, based on a bioassay carried out on guinea-pig phreno-diaphragmatic preparations *in vitro*; nevertheless, the strain of *G. impudicum* (10B) from Fusaro Lagoon gave negative results with mouse bioassays (Williams, 1984) and no PSP toxins were detected by HPLC.

G. impudicum shows the pigment pattern usually present in photosynthetic dinoflagellates (Jeffrey *et al.*, 1975) and it *is* very similar to what was previously observed in *G. catenatum* by Hallegraeff *et al.* (1991). This pattern is clearly different from the pigment composition of other *Gyrodinium* and *Gymnodinium* species such as *Gyrodinium aureolum* Hulburt (refers to *Karenia mikimotoi*) and *Gymnodinium galatheanum* Braarud (Refers to *Karlodinium micrum*), recently included by Johnsen & Sakshaug (1993) as representatives of a group of dinoflagellates containing chlorophylls a, c_3 and c_2, and fucoxanthin and its derivatives 19' butanoyloxyfucoxanthin and 19' hexanoyloxyfucoxanthin.

Costas & López-Rodas (1994) and Aguilera *et al*. (1995) used several strains of *G. impudicum* (as *Gymnodinium* sp. and *Gyrodinium* sp. respectively) and 4 of *G. catenatum* to test binding of nine FITC-labelled lectins on these dinoflagellates. They found that WGA lectin from wheat germ (Serva Feinbiochemica GMBH & Co.) specifically bound with *G. catenatum* but not with *G. impudicum*. The similarity between *G. impudicum* and *G. catenatum* makes it very difficult to distinguish unless one has access to both species. This difficulty is magnified in fixed material, as in most monitoring programmes. In some cases the use of FITC-labeled lectins can be useful Costas & López-Rodas (1994) and Aguilera *et al*. (1995).

If the Greek and Japanesse reports were atributable to *G. impudicum*, their observations agree well with the ability of *G. impudicum* to bloom in warm, saline and polluted waters. Friligos & Gotsis-Skretas (Friligos & Gotsis-Skretas, 1989) reported a red tide of *Gymnodinium catenatum*-like species (presumably *G. impudicum*) in Pagassitikos Gulf (Greece) at 25°C and 37.2 PSU, and related it to pollution and eutrophication in the station nearest to the city of Volos. Iwasaki (1971) reported that decomposed matters or the extracts of animal tissues, yeast or casein, remarkably increase the growth of *G. impudicum* (as *P. schwartzi*) in laboratory cultures. Ishio *et al.* (1977), after experiments with the same strain (as *Gyrodinium* sp.) reported the incorporation of organic particles from the culture medium by the cells. Fraga *et al*. (1989) have related the ability of some dinoflagellates to form chains as an evolutionary adaptation to red tides. The ability of *G. impudicum* to form massive blooms of cells in chains agrees well with this hypothesis. The ecophysiological significance of mucus production and the two

kinds of behaviour observed in culture, (active swimming and non motile) are not yet clear, and will be the subject of further studies.

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DIFFERENCES BETWEEN TWO CHAIN FORMING, ATHECATE, RED TIDE DINOFLAGELLATES: *GYMNODINIUM CATENATUM* AND *GYMNODINIUM IMPUDICUM*.

Gymnodinium impudicum is a naked dinoflagellate that typically form four celled chains, although longer chains can be also observed, having 16 cells the longer observed. They have different shapes according to the relative position they have in the chain, or if they are in singles. The anterior cell of a chain has a pointed epicone and a truncated hypocone, which holds the nucleus. The cells inside the chain have both extremes truncated and the nucleus is central. The posterior cells of a chain have a truncated epicone and a pointed hypocone, being the nucleus towards the epicone. Single cells have intermediate characteristics: both extremes are pointed and the nucleus is central. The size of the cells is smaller than *Gymnodinium catenatum* and many times increases considerably towards the posterior end of the chain. The cingulum is deeply marked and has a displacement ranging between 1/3 and 1/5 of the length of the cell, reason why *G. impudicum* was included firstly in genus *Gyrodinium*. The longitudinal sulcus penetrates the epicone towards the apex which is surrounded by the dorsal side from left to right to form an acrobase. In this species the acrobase is just a prolongation of the sulcus, not like in *G. catenatum* in which they appear as independent morphological structures. The connection of two cells is through the dorsal side of the acrobase as in *G. catenatum*. The hypocone is slightly smaller than the epicone and with a convex silhouette, contrary to *G. catenatum* in which the hypocone is slightly bigger than the epicone and has most of the times a slightly concave silhouette on healthy cells. It has numerous rounded chloroplasts bigger and less elongated than those of G. catenatum, having similar pigment patterns, common to most dinoflagellates. When *G. impudicum* is in great concentrations, either in cultures on in natural blooms, it appears embedded in mucus, and at least in culture, it has a very peculiar behaviour: most of the time it is motionless, but when it swims, it is very fast.

In extracts of cells of *G. impuducum* cultured simultaneously at the same conditions as *G. catenatum*, no toxins were detected while in the later species were clearly detected. Similar analysis on extracts obtained from a natural bloom in Valencia gave the same result. Mice bioassays were also negative.

Summarizing, the main differences between *G. catenatum* and *G. impudicum* (Fig. 26) are: Average size, chain length, cingular displacement, shape of the acrobase, hypocone

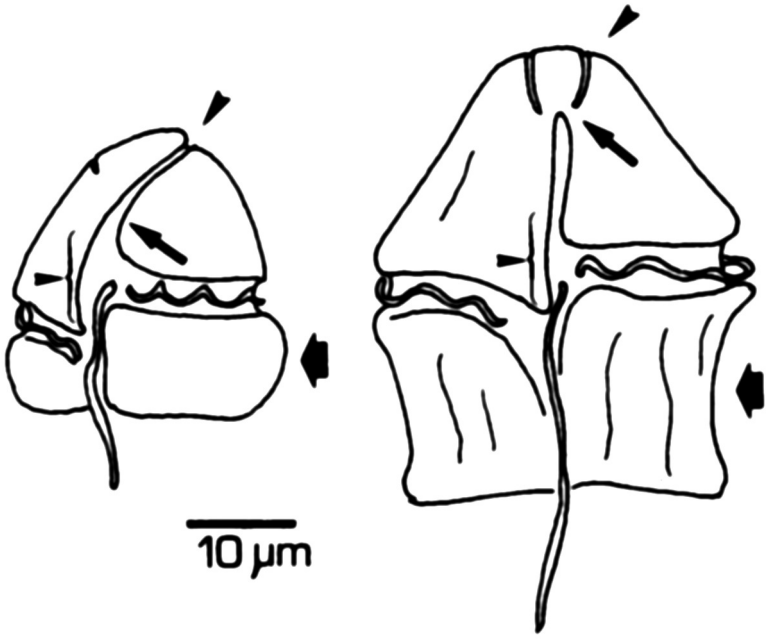

Fig. 26. Sketches of *Gymnodinium impudicum* (left) and *Gymnodinium catenatum* (right) drawn at the same scale. Arrow heads show acrobase and thick arrows hypotheca silhouettes.

silhouette, chloroplasts, toxicity, swimming behaviour, mucus production and sterols composition.

After this work was done, ribosomal DNA of *G. catenatum* and *G. impudicum* was sequenced showing that these to species were genetically clearly different (Daugbjerg et al., 2000). The cysts of *G. impudicum* were also described (Kobayashi *et al.*, 2001) and were different from the microreticulate cysts of *G catenatum* (Bravo, 1986, Anderson et al., 1988, Blackburn *et al.*, 1989).

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CAPÍTULO 2

GÉNERO *COOLIA*

COOLIA CANARIENSIS SP. NOV. (DINOPHYCEAE), A NEW NON TOXIC EPIPHYTIC BENTHIC DINOFLAGELLATE FROM THE CANARY ISLANDS

ABSTRACT

A new photosynthetic dinoflagellate species, *Coolia canariensis* Fraga sp. nov. is described based on samples taken from tidal ponds on the rocky shore of the Canary Islands, NE Atlantic Ocean. Its morphology was studied by light and scanning electron microscopy. It is almost spherical and has a thick smooth theca with many scattered pores. Plate 4' is biggest of the epithecal plates and 6" is twice as wide as it is long. Phylogeny inferred from the D1/D2 regions of the large subunit nuclear rDNA of three strains of *C. canariensis* and several strains of other *Coolia* species *C. monotis*, *C.* sp.showed that *C. canariensis* strains clustered in a well supported clade distinct from the other species. No toxins were detected using mouse bioassay, LC-FLD or LC-MS. Its pigment composition is of the peridinin type of dinoflagellates. This work increases the number of species of this genus and helps to better define the type species of the genus, *Coolia monotis* Meunier. Together with this new species, many other strains of *C. monotis* from the Atlantic Ocean and Mediterranean Sea have been analyzed for toxin presence and no evidence of toxin production related to YTXs (yessotoxins) was found, as was previously suggested for *C. monotis* from Australia.

INTRODUCTION

Benthic and epiphytic dinoflagellates have attracted much attention from researchers, since some of them were associated with ciguatera fish poisoning, a syndrome caused by eating toxic fish from tropical areas (Yasumoto *et al.*, 1977). Although the responsible toxins have only been found in dinoflagellates of the genus *Gambierdiscus* Adachi & Fukuyo, other toxic species of different genera often occur associated in a benthic dinoflagellate assemblage, such as *Ostreopsis* Schmidt, *Prorocentrum* Ehrenberg, *Amphidinium* Claparède & Lachmann, and *Coolia* Meunier (Fukuyo, 1981) and it has been suggested that toxins from these other species may contribute to the complexity and variability of symptoms observed in people affected by ciguatera (Tindall & Morton,

1998). However differential amounts of ciguatoxins ingested, the chemical profile of ciguatoxins and individual susceptibility may also account for this variability (Lewis, 2001). *Ostreopsis* spp. produce palytoxin analogs (Lenoir *et al.*, 2004, Usami *et al.*, 1995) and these toxins have been identified from poisonous fish (Kodama *et al.*, 1989, Onuma *et al.*, 1999), but this is usually considered an intoxication different from ciguatera. Recently, a new concern about benthic dinoflagellates was raised when *Ostreopsis ovata* Fukuyo was associated with human illness among people exposed to marine aerosols in Italy (Ciminiello *et al.*, 2006).

One strain of *C. monotis* Meunier (Meunier, 1919) was previously reported to produce a toxin named cooliatoxin (Holmes *et al.*, 1995). Since this report other strains of *C. monotis* have been examined but with no toxins detected (Riobó *et al.*, 2004) and other *C. monotis* extracts were found to be not toxic to mice (Rhodes *et al.*, 2000). In the study of (Holmes et al., 1995) it was suggested that cooliatoxin may be an analog of yessotoxin based upon similar bioassay symptoms in mice and mass spectrometry, but this has never been confirmed.

Coolia spp. was a mono-specific genus when it was first described in the early 20th century until two new species of *Coolia* were described at the end of the century: *Coolia tropicalis* Faust (Faust, 1995) and *Coolia areolata* Ten-Hage, Turquet, Quod & Couté (Ten-Hage *et al.*, 2000). Previously, due to its similarity with genus *Ostreopsis* Schmidt, *C. monotis* was transferred to the genus *Ostreopsis* (Lindeman, 1928) and this was accepted by (Schiller, 1937). (Biecheler, 1952) subsequently transferred it to the genus *Glenodinium* based on the tabulation of the hypotheca, but after the detailed morphological study of (Balech, 1956), the genus name *Coolia* was reinstated. Recent phylogenetic analyses based on the ITS 5.8S rDNA sequences (Penna *et al.*, 2005) and (D1/D2) LSU rDNA (Dolapsakis *et al.*, 2006) confirmed the separation of the two genera as stated originally by (Meunier, 1919). In the study of (Penna et al., 2005) it was found that under the specific name of *C. monotis* two different clades were present: an Asian clade, which also includes a strain from Florida, and a European clade. Based on the coincidence of the morphology of the European strains with the original description of *C. monotis,* (Meunier, 1919), and the fact that two of the sequenced strains are from the Netherlands, which is close to the species type locality, Nieuport, Belgium, the European clade can be considered the original species of *C. monotis,* while the Asian clade corresponds to another recently described species, *C. malayensis* Leaw, P.-T. Lim et Usup (Leaw *et al.*, 2010). Neither the sequences of *C. areolata* nor *C. tropicalis* have been published.

In this study, a new species *Coolia canariensis* sp. nov. is described on the basis of morphology, genetic sequencing, pigment and toxin composition of three strains isolated in the Canary Islands. This species turns out to be morphologically different from the

other species of *Coolia* already described and genetically different from other *Coolia* strains sequenced.

MATERIALS AND METHODS

Source of specimens and culture conditions

Samples were collected at Punta Hidalgo, a rocky shore on the north coast of Tenerife, in the Canary Islands' archipelago in the NE Atlantic Ocean (28º 34'N, 16º 19'W). Samples of small mixed seaweeds were collected from tidal pools on the rocks during low tide, placed in plastic bottles and shaken. Afterwards, the gross materials were removed and the remaining seawater was used for cell isolation. Isolation was carried out by a capillary pipette with the aid of a Zeiss Invertoscop D microscope (Carl Zeiss AG, Germany) and isolated cells were incubated in 96 microwells plates in full strength L1 medium (Guillard & Hargraves, 1993) made with seawater from Ría de Vigo with a salinity adjusted to 34 psu and incubated at 24 ºC and a photon irradiance of 90 µmol $m^{-2} \cdot s^{-1}$ of PAR measured with a QSL-100 irradiameter (Biospherical Instruments Inc. San Diego, CA, USA) and at a 14:10 L:D photoperiod. The cultures are deposited at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo. These and other cultures used in this study and obtained from different sources are listed in Table 1.

Light microscopy

The cultured cells were observed alive or fixed with formalin. For plate pattern identification the cells were stained with Fluorescent Brightner 28 (Sigma, St Louis, MO, USA) following a modified (Fritz & Triemer, 1985) technique. Others were dissected, squashing the cells by pressing the cover slip over them and sometimes with the aid of sodium hypochlorite and stained with the chloral hydrate Imamura and Fukuyo method (Taylor, 2004). The nuclei were stained using SYBR Green (Molecular Probes, Eugene, OR, USA) following the method of (Figueroa & Bravo, 2005). Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence. The photographs were taken with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera. When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and were then overlapped as different layers using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The areas of each layer that were out of focus were manually erased and the layers flattened to get a final image with an enhanced depth of field. Cell size was measured by LM with the aid of an ocular micrometer.

TABLE 1. Sample locations, origin, source, EMBL accession numbers of the different strains used in this study

Species	**Strain**	**Sampling locality**	**Isolator**	**EMB Accession no.**
Coolia canariensis	VGO787	NE Atlantic, Canary Islands, Spain	S. Fraga	AM902738 [a]
Coolia canariensis	VGO786	NE Atlantic, Canary Islands, Spain	S. Fraga	AM902737[a]
Coolia canariensis	VGO775	NE Atlantic, Canary Islands, Spain	S. Fraga	AM902739[a]
Coolia tropicalis	CCMP1744	Caribbean Sea, Belize	S.L. Morton	AM902741[a]
Coolia tropicalis	VGO923	W Pacific, Manado, Indonesia	S. Fraga	AM902740[a]
Coolia monotis	CM7C	Mediterranean, Catalan Sea, , Spain	S. Fraga	AM902745[a]
Coolia monotis	VGO782	Mediterranean, Saronikos Gulf, Greece	S. Fraga	AM902746[a]
Coolia monotis	VGO783	Mediterranean, Saronikos Gulf, Greece	S. Fraga	AM902747[a]
Coolia monotis	SZN-268	Mediterranean, Naples, Italy,	M. Montresor	AM902748[a]
Coolia monotis	VGO831	Mediterranean, Almería, Spain	S. Fraga	AM902744[a]
Coolia monotis	CBA-1	Mediterranean, Genova, Italy	A. Penna	AM902742[a]
Coolia monotis	RIKZ4	North Sea, The Netherlands	L. Peperzack	AM902749[a]
Coolia malayensis	CCMP1345	Caribbean Sea, Florida, USA	J. Bomber	AM902743[a]
Coolia malayensis	CmPL01	Malaysia	G. Usup	AF244942[b]
Coolia cf. *monotis*	CAWD39	Kaitaia, Northland, New Zealand	L. Rhodes	U92258[c]

[a] This study

[b] Usup et al

[c] Rhodes, L.

Sample preparations for SEM

Five mL of exponentially growing cultures were fixed with GTA at a final concentration of 4%. After two hours at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH. After being air dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a Phillips XL30 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

Nomenclature

In this study, the Kofoid tabulation system (Kofoid, 1909) as described in (Fensome *et al.*, 1993), was followed to name the plates therefore allowing comparisons with other descriptions. The study of possible plate homologies among different dinoflagellate genera is not among the objectives of this study, so all the plates of the epitheca in contact with the cingulum were named precingular plates. The terms «length» as apical/antapical distance and «width» as transdiameter were used for the dimensions (Taylor, 2004).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from approximately 20-30 mL culture in logarithmic growth phase using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The D1/D2 regions of the LSU rDNA were amplified by using primers D1R and D2C by (Scholin *et al.*, 1994). Genomic DNA (1 ng) was amplified in 50 µl reaction mix containing 50 µM of each dATP. dTTP, dCTP and dGTP; 0.3 µM of each primer; 4mM $MgCl_2$; 1x reaction buffer (Diatheva, Fano, Italy): and 1.0 U Hot Rescue DNA Polymerase (Diatheva, Fano, Italy). Thermocycling was as follows: 10 min initial denaturation at 95 °C; 35 cycles of 1min at 94 °C, 1 min at 50 °C, and 2.5 min at 72 °C; with a final elongation step of 7 min at 72° C. Three PCR amplified products of the D1/D2 of the LSU gene were pooled, purified, and then directly sequenced or cloned for sequence analyses. The amplified PCR fragments were cloned in the vector pDrive Cloning Vector (Qiagen, Valencia, CA) and sequenced. Nucleotide sequencing was performed using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method was used according to the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit, Perkin Elmer Corp., Foster City, CA). Each clone was sequenced in both directions in order to remove ambiguities. The sequences were deposited in the EMBL Bank and are listed in Table 1. *Phylogenetic analyses.* Sequence alignment was carried out with SAM software (Karplus *et al.*, 1998) and subsequently checked by eye. The D1/D1 regions of the LSU gene of *Coolia* spp. isolates were also aligned with a few other sequences of

D1/D1 regions of the LSU gene of *Coolia* species from GenBank (Table 1). Phylogenetic relationships, based on the D1/D2 LSU rDNA, were inferred using the Maximum Parsimony, MP, Maximum Likelihood, ML and Bayesian, BI methods. The sequence of *O. ovata* (AF244940) was used as an outgroup. The best fit model of nucleotide substitution for the phylogenetic analyses was the Akaike Information Content implemented in Modeltest 3.06 (Posada & Crandall, 1998). The General Time Reversible model (Lanave *et al.*, 1984) with a gamma-shaped distribution for among-site rate variation (alpha value of the gamma distribution equal to 0.5), was selected and used in the MP, ML and BI analyses. In the ML analyses, the branch-swapping algorithm with 100 random additions in the TBR (Tree Bisection Reconnection) option was used. Robustness of the phylogenetic trees, generated by MP, ML and BI was tested by using the non-parametric bootstrap with 1,000 replicates, respectively. The above analyses were performed with the software packages PAUP* ver. 4.0b10 (Swofford, 2000). The Bayesian Inference analyses were carried out with MrBayes ver. 3.0b4 (Huelsenbeck & Ronquist, 2001) using the General Time Reversible model with a gamma-shaped distribution for among-site rate variation; the Monte Carlo Markov Chain length was 2,000,000 generations with a sampling frequency of 100 generations. Log-likelihood values for sampled trees were stabilized after almost 200,000 generations; the last 20,000 trees were used to estimate Bayesian posterior probabilities (Bpp), while the first 2,000 were discarded as burn in. Posterior probabilities correspond to the frequency at which a node is found in the post burn in tree. Standard and molecular diversity indices were inferred based on the aligned sequence data set and calculated with Arlequin ver. 2.0 software (Schneider *et al.*, 2000).

Pigment analyses

Cultures were examined by light microscopy before carrying out HPLC pigment analysis to ensure the cells were healthy and with good morphology. Cells were harvested 3 hours into the light cycle from cultures in exponential growth phase. Ten mL of culture were filtered onto Whatman GF/F filters under reduced pressure. Filters were frozen immediately at -25 ºC, and analyzed within 12 hours. Frozen filters were extracted under low light in Teflon-lined screw capped tubes with 5 mL 90% acetone using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 minutes in an ultrasonic bath. Extracts were then filtered through 25 mm diameter syringe filters (MFS HP020, 25 mm, 0.20 mm pore size, hydrophilic PTFE,) to remove cell and filter debris. An aliquot (0.5 mL) of methanol extract was mixed with 0.2 mL of water and 200 ml was injected immediately. This procedure avoids peak distortion of early eluting peaks (Zapata & Garrido, 1991) and prevents the loss of non-polar pigments prior to injection in an HPLC system. Pigments were separated using a Waters (Waters Corporation, Milford, MA) Alliance HPLC System consisting of a 2695 separations

module, a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface. Pigment separation was performed using the HPLC method of (Zapata *et al.*, 2000), with a reformulated mobile phase A. The column was a C_8 monomeric Waters Symmetry (150 x 4.6 mm, 3.5 mm particle-size, 100 Å pore-size;). Eluent A was methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v). Eluent B was methanol: acetonitrile: acetone (20:60:20 v/v/v). Elution gradient was as follows: (time: %B) t_0: 0%, t_{22}: 40%, t_{28}: 95%, t_{37}: 95%, t_{40}: 0%. Flow rate 1.0 mL·min^{-1} and column temperature was 25 °C. Solvents were HPLC grade (Romil-*SpS*^{TM}); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After checking for peak purity, spectral information was compared with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see (Jeffrey & Wright, 1997).

Toxin analyses

For yessotoxins (YTXs) analyses, 50 mL aliquots of each strain were harvested and were filtered through 1.4 µm GF/C glass fibre filters (25 mm Ø) (Whatman, Maidstone, England). YTXs in both, medium and cells, were extracted and determined by LC-MS and by LC-FLD analysis following a previous report (Paz *et al.*, 2004). LC-FLD analysis was carried out by a system equipped with a Hitachi L-6200 A pump, a Jasco FP-920 fluorescence detector, a Hitachi AS-4000 autosampler and a Lichrospher 100 RP18 5 µm (4.6 x 125 mm) column at 35 ºC. A mobile phase of 100 mM ammonium acetate, pH 5.8:MeOH (3:7) at a flow rate of 0.75 mL·min^{-1} was used. The excitation and emission wavelengths were 370 nm and 440 nm, respectively (Paz et al., 2004). For LC-MS separation an Xterra MS C18 5 µm (2.1 x 150 mm) column at 35 ºC, 2 mM ammonium acetate (pH 5.8) (A) and MeOH (B) as mobile phase, were used. A gradient elution (40 to 30% A in 5 min, 30 to 20% A in 5 min, followed by 5 min with 20% A, then 20 to 0% A in 5 min and 0% A for 2 min) was used. A flow rate of 0.20 mL·min^{-1} was used. Mass spectral analyses were performed using an ion trap mass spectrometer, Thermo Finnigan LCQ-Advantage, equipped with an electrospray ionization (ESI), in negative ion mode. ESI was performed with a 4.5 kV spray voltage and 120 °C capillary temperature, flow 60 mL·min^{-1} for sheath gas and 20 mL·min^{-1} for auxiliary gas. Full scan data were acquired from m/z 500 to 2000. Extracted ion chromatograms for possible YTXs were performed at m/z 1141 (YTX), 1061 (desulfoYTX), 1155 (homoYTX), 1173 (carboxyYTX), 1187 (carboxyhomoYTX), 1047 (Ciminiello *et al.*, 2002a) (noroxoYTX) and 1273 (G-YTXA) for ions [M-2Na+H]- (Ciminiello *et al.*, 2003, Draisci *et al.*, 1999, Souto *et al.*, 2005) Subsequent LC-MS2 analysis for relevant peaks at m/z 1187 and 1065 were performed

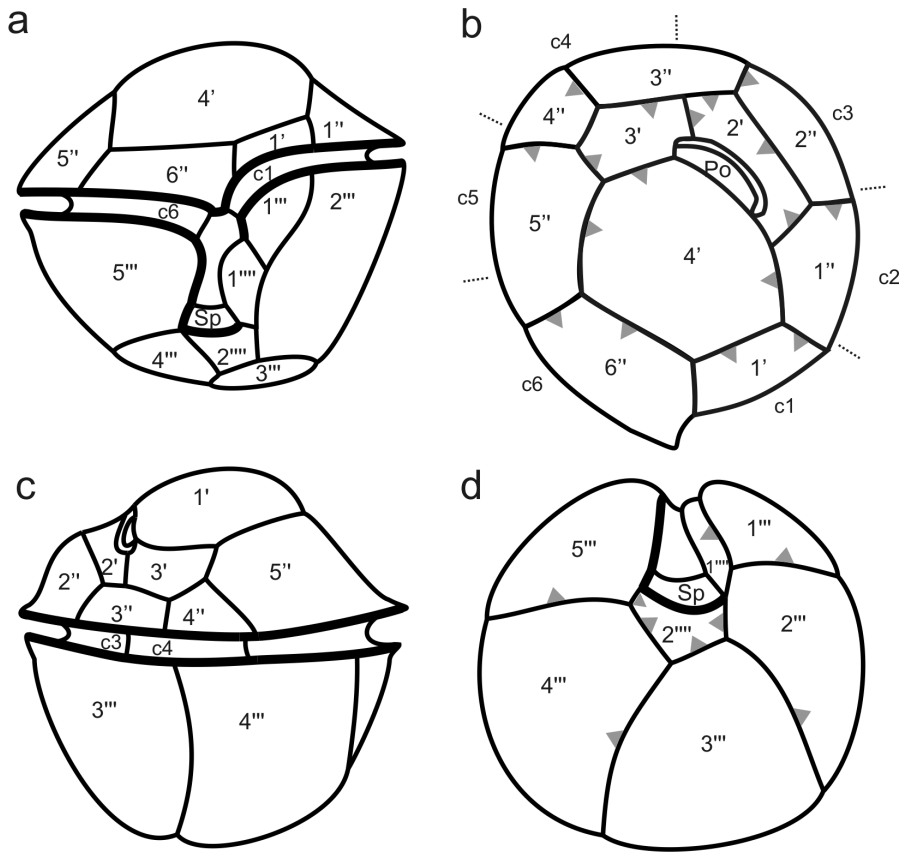

Fig. 1. Ink drawings of *C. canariensis*. (a) Ventral view. (b) Apical view. (c) Dorsal view. (d). Antapical view. Grey arrows indicate direction of plate overlap.

by applying a Collision Energy (C.E.) of 40%. A YTX standard solution provided by the Institute of Environmental Science and Research Limited (New Zealand) was used for identification of possible YTX and the toxic strain GG1AM of *Protoceratium reticulatum* was used as a reference.

RESULTS

Coolia canariensis *Fraga sp. nov.*

Fig. 1, a-d, Fig. 2 ,a-f, Fig. 3, a-d, Fig. 4, a-e, Fig. 8, a-b

Cellula quasi spherica, 27.2-38.4 µm longa et 25.6-40 µm lata. Forma nuclei aequatorialis littera U similis est, cuius extrema ventraliter directa sunt. Formula laminarum: Po, 4', 6'', 6c, ?s, 5''', 2''''. Theca crassa levisque est et permultos poros orbiculatos habet, praeter prima

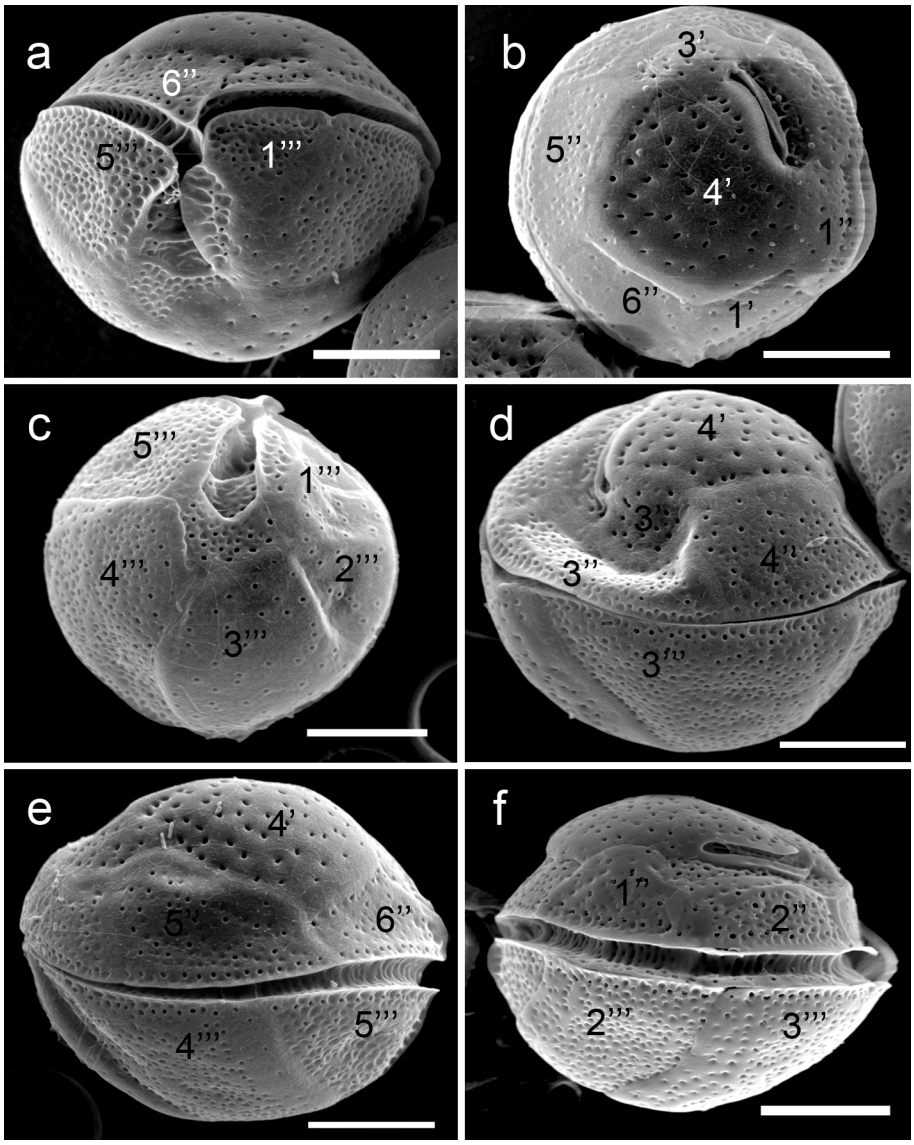

Fig. 2. SEM images of *C. canariensis*. (a) Ventral view. (b) Apical view. (c) Antapical view. (d). Dorsal view. (e). Right side view. (f). Left side view. All scale bars: 10µm.

apicalis lamina, maior epithecae, quae habet eos ovales. Septima lamina precingularis bis lata quam longa est. Cingulum profundum est et pene dimotum. Photosynthetica est et multos cloroplastos habet cum peridinina ut principali carotenoide. Toxica non est.

Cells almost spherical 27.2-38.4 µm long and 25.6-40 µm wide with an equatorial U-shaped nucleus with the extremes pointing ventrally. Plate formula: Po, 4', 6'', 6c, ?s, 5''', 2''''. Thecal plates are thick and smooth with numerous circular pores. The first

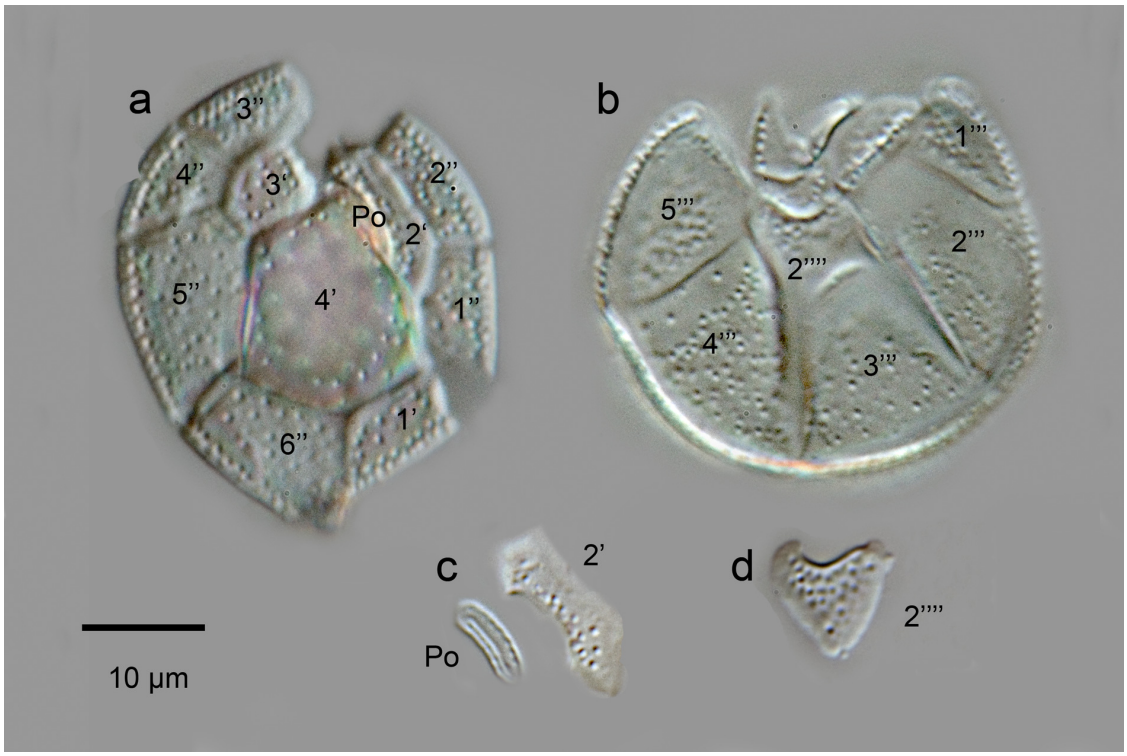

Fig. 3. LM images of *C. canariensis*. (a) Squashed epitheca. (b) Squashed hypotheca. (c) Detail of plates Po and 2’. (d) Detail of plate 2’’’’. All scale bars: 10µm.

apical plate is the biggest of the epitheca and has oval pores. The seventh precingular plate is twice as wide as long. Cingulum deep and slightly displaced. The species is photosynthetic and has many chloroplasts having peridinin as a major carotenoid. It is non-toxic.

Holotype: Figure 1 obtained from strain VGO787 deposited in the Culture Collection of Harmful Microalgae (CCVIEO) of the Centro Oceanográfico de Vigo of the Instituto Español de Oceanografía, Vigo, Spain.

Type locality: Punta Hidalgo, (28º 34.0’ N, 16º 19.8 ’W) Tenerife, Canary Islands, NE Atlantic Ocean.

Habitat: Marine, associated to seaweeds in a rocky tidal pool.

Etymology: The epithet refers to the Canary Islands, the place where *C. canariensis* was first observed and the origin of the cells used in this description.

Other specimens examined: Clonal strains VGO775, VGO780 and VGO786, isolated from the same field sample as the holotype and deposited in the Culture Collection of

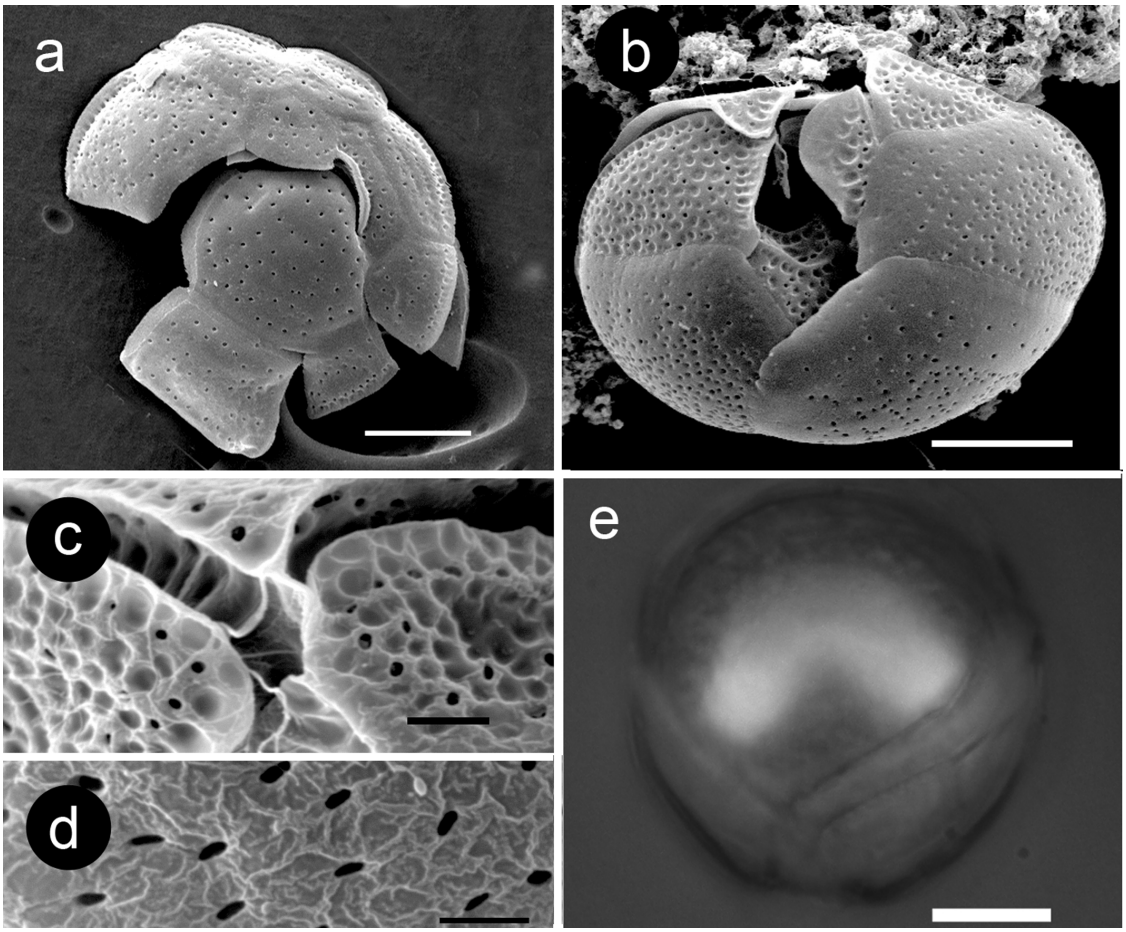

Fig. 4. *C. canariensis*. (a) SEM image of squashed epitheca. (b) SEM image of ventral side of hypotheca showing plates overlapping. (c) SEM image of cingular displacement. (d) SEM image of thecal pores in plate 1'. (e) Mixed transmitted and epifluorescence light microscopy image of the U shaped nucleus viewed from the ventral side of the epitheca. Scale bars: 10 µm in a, b and e;

Harmful Microalgae (CCVIEO) of the Centro Oceanográfico de Vigo of the Instituto Español de Oceanografía, Vigo, Spain.

Morphological description

Cells of *C. canariensis* are almost spherical 27.2-38.4 µm long (average 32.7 µm) and 25.6-40 µm wide (average 32.8 µm); the ephitheca is slightly smaller than the hypotheca (Figs. 1a, 2a). The plate formula is Po, 4', 6'', 6c, ?s, 5''', 2''''. It was not possible to observe all the sulcal plates. Po is elongated and is 8µm long (Figs. 2b, 3c). 4' is hexagonal and the biggest plate of the epitheca and it has a central position being surrounded by 1', 1", 2' Po, 3', 5" and 6" (Figs. 2b, 3a). Plate 2' is very small, hexagonal,

elongated and embraces Po along its left and dorsal sides (Figs. 2b, 3a and c, 4a). It is overlapped by 1", 2", 3" and 3', and overlaps 4' (Fig. 4a). Being so small and overlapped by so many plates it usually appears forming a depression together with Po (Figs. 2b and d). Plate 3' is pentagonal and contacts 4', Po, 2' 3", 4"and 5" (Figs. 2d, 3a). The apical pore plate Po and 2', which shares the left side of 3' with Po, does not allow contact between 2" and 3'. Plates 1', 2" and 4" are small and four sided. 1" and 3" are small and five sided (Figs. 3a, 4a). Plate 5" is very short and wide in a way that forces 3' to be very dorsally situated (Fig. 2d). Plate 5" is the biggest of the precingular plates and is 5 sided (Figs. 2a and d, 3a, 4a). Plate 6" is four sided and follows in size 5" (Figs. 2a, b, e and 4a). First postcingular plate 1''' is triangular and together with 6" defines the cingular displacement (Fig. 2a). Plates 2''', 3''', 4''' and 5''' are big and elongated towards the antapex (Figs. 2c, 3b, 4b). Plate 1'''' is like a rounded wing over the anterior part of the sulcus (Figs. 2a, c and 4b). Plate 2'''' is small and five sided but having a general appearance of triangular (Fig. 3d) and in its connection with S.p. has an abrupt discontinuity on the cell surface making the sulcus well limited in its posterior end (Figs. 2a, c and 4b). Thecal plates are thick and smooth, and are covered with scattered pores although some postcingular plates, especially 1''' and 5''', may be ornamented with small pits, in the center of which there is sometimes a pore (Fig. 4c). The pores of plate 4' are oval (Figs.2b and 4d) while those of other plates are round. Plates 3''' and 4' are smoother and apparently thicker than the other plates (Figs. 2a, b, c and 4b). The sides of the plates that overlap contiguous plates lack pores facilitating their observation when the sutures among them are very faint. They may show growth bands without pores that can be very wide. The cingulum is very deep, descends about two cingulum widths, and has 6 plates. The cingular plates have a striated ornamentation that makes difficult to see their sutures with TEM and hence disection under LM is neccessary to solve them. Cingular plates c1 and c2 are the smallest and c6 is the longest. Sulcus is very deep and short with the anterior part partially hidden by 1'''' (Figs. 2a, c and 4b).

The nucleus is U shaped and is horizontal and dorsally located just beneath the cingulum with the tips facing the ventral side (Fig. 4e). The species has numerous chloroplasts.

Distribution and ecology

C. canariensis was found associated with *Gambierdiscus* sp., *Prorocentrum arenarium*, *Prorocentrum lima, O. ovata* and *Amphidinium* sp. as epiphytic on a mixture of small seaweeds in rocky tidal pools on the north coast of the island of Tenerife. It is notable that this is not a protected area and it is affected by the strong dominant trade winds with a seawater temperature ranging from 18 to 26 ºC and a yearly average of

about 21 ºC (Borges *et al.*, 2004). When in culture, *C. canariensis* produce mucus but not as much as *C. monotis*.

Phylogenetic analyses of Coolia species

The size of the D1/D2 regions of LSU rDNA was 777 bp. The final alignment with *O. ovata* as outgroup was 772 bp in length (C 13.12%, T 33.66%, A 32.03% and G 21.19%) with 566 polymorphic sites and with 271 transitions and 268 transversions. As substantially identical tree topologies were obtained by the MP, ML and Bayesian Inference methods, only the Bayesian phylogenetic tree is presented (Fig. 5). The D1/D2 of LSU rDNA phylogeny showed that within genus *Coolia* three different genetic lineages were present. The first lineage comprised two strains originally isolated from Indonesia and Belize. The second grouping included isolates belonging to the new species of *C. canariensis*. The last grouping was formed by two sister clades: one including *C. monotis* from different European localities and the other consisting of *C. monotis* and *Coolia malayensis* from different geographical areas, New Zealand, Malaysia and Florida. These groupings were well supported by high bootstrap values (for MP and ML) and posterior probability of the BI inferenc*e*.

Pigments

C. canariensis strains VGO775 and VGO778 show the pigment profile characteristic of peridinin-containing dinoflagellates (Fig. 6) (Jeffrey *et al.*, 1975, Liaaen-Jensen, 1998). Chlorophyll c_2 was the major accessory chlorophyll (chl c_2/chl a = 0.35), in addition, traces of MgDVP were found (MgDVP/chl a = 0.06) but chl c_1 was not detected. Peridinin was the major carotenoid (65.4% of total carotenoids) followed in relative importance by diadinoxanthin (18.8 %), dinoxanthin (6.5 %), peridininol (4.2%), â,â-carotene (1.4 %) and diatoxanthin (0.3 %). A carotenoid with retention time and spectrum similar to *all-trans*-neoxanthin ($\ddot{e}_{max}$: 416, 442, 471 nm) was detected at trace level (0.7%) eluting just ahead of an unknown carotenoid whose quantitative contribution was 3.7% of total carotenoids. The absorption spectrum (l_{max}: (424), 448, 477 nm) of the unknown carotenoid resembles diadinoxanthin, although it is more polar.

Toxins

Characteristic double peaks for derivatized YTX were not detected in the any of the strains VGO775, VGO780, VGO786 and VGO787 of *C. canariensis* analysed by LC-FLD. In addition, several *C. monotis* strains (CM2V, CM6V, VGO782, RIKZ4, CCMP1345 and VGO858) were analysed by LC-FLD but none of them showed the double peak of YTXs while these two peaks were clearly observed in strain GG1AM of *P. reticulatum*.

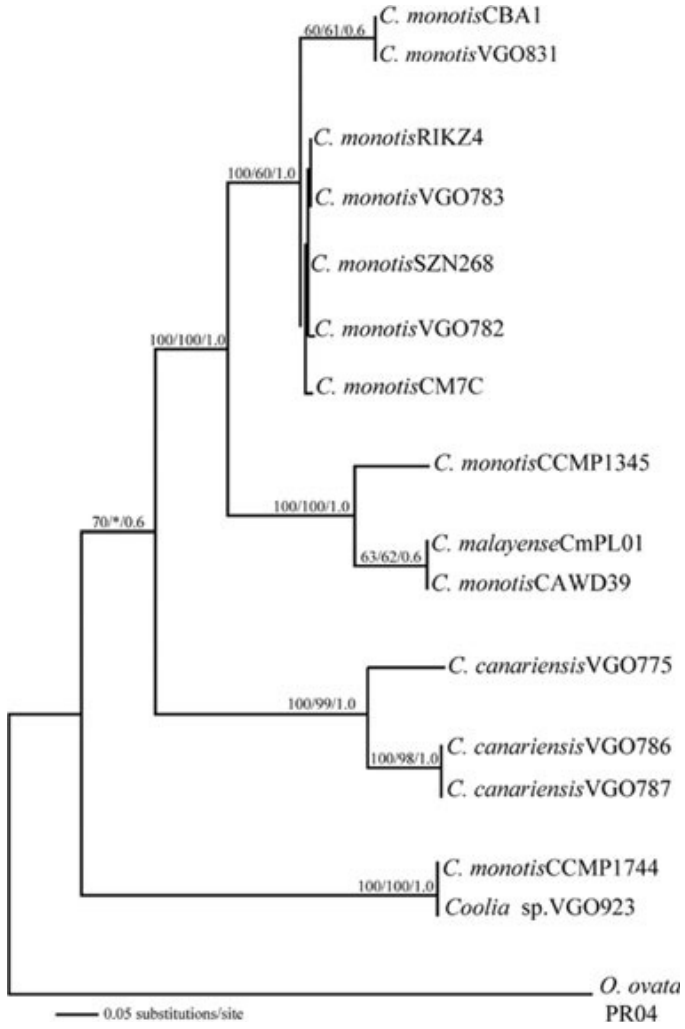

Fig. 5. Bayesian phylogenetic tree of the genus *Coolia* based on the D1/D2 regions of the LSU gene sequences. Numbers on the major nodes represent from the left to the right, MP (1,000 replicates), ML (1,000 replicates) bootstrap values and Bayesian posterior probability values. Only bootstrap and posterior probability values > 50% and 0.5 are shown, respectively. Asterisk symbol at the major node represented bootstrap values < 50%. The tree is rooted using *O. ovata* PR04 (AF244940) as outgroup.

Complementary LC-MS analysis at m/z characteristic for known YTXs confirm the absence of YTX or YTX analogs in all the analysed strains of *C. canariensis*. Nevertheless, the VGO780 and the VGO775 strains of *C. canariensis* showed a peak for the ion at m/z 1187 (Fig. 7a), with the same mass as that of carboxyhomoYTX, a YTX analog produced by metabolism in shellfish but not in algae (Ciminiello *et al.*, 2000). LC-MS2 fragmentation

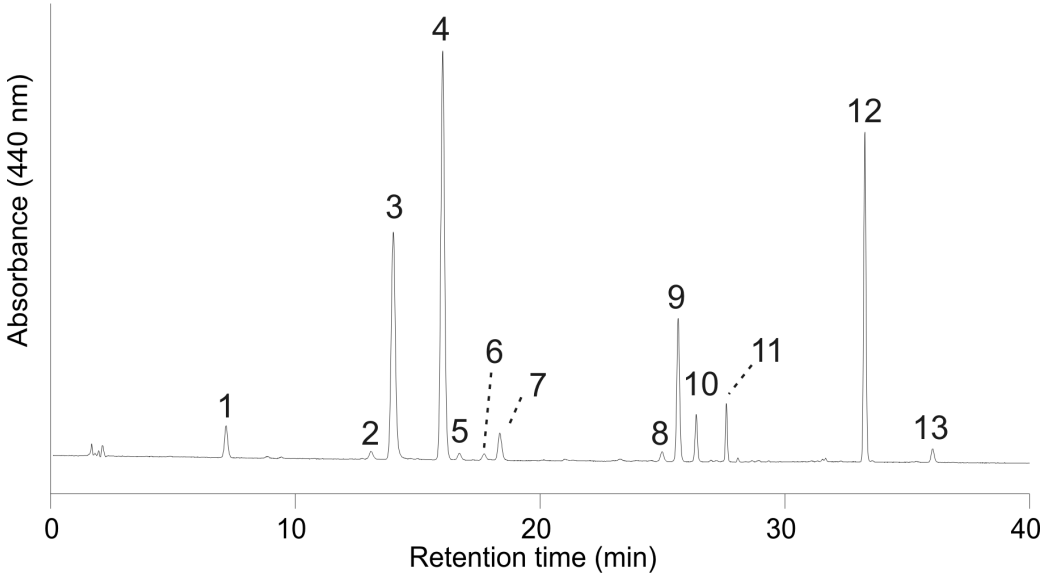

Fig. 6. HPLC chromatogram of *C. canariensis* strain VGO787. Peak identification: (1) Peridininol, (2) Divinyl protochlorophyllide (MgDVP), (3) Chl c_2, (4) Peridinin, (5) Peridinin-like, (6) *all-trans* neoxanthin-like, (7) Unknown carotenoid 448 nm, (8) Diadinochrome, (9) Diadinoxanthin, (10) Dinoxanthin, (11) Diatoxanthin, (12) Chlorophyll *a*, (13) beta, beta-carotene. Detection by absorbance at 440 nm.

of this ion was performed (Fig. 7a), but the daughter ions scheme was not consistent with the characteristic fragmentation of the YTX side chain observed in the *P. reticulatum* strain used as a reference (Fig. 7b) (Ciminiello et al., 2003, Ciminiello *et al.*, 2002b), in which the first fragmentation daughter ion (m/z 1061) differs from the pattern ion in 80 m/z units due to the lost of a sulphate group. Subsequent fragmentation for the daughter ion at m/z 1061 and also for the desulfoYTX analogs gave characteristic ions at m/z 925 and 855 (Fig. 7b), therefore this peak was discounted as a possible YTX analog. The CM2V, VGO858, RIKZ4 and CCMP1345 strains of *C. monotis* displayed a peak at m/z 1067 (Fig. 7c), similar to that of desulfoYTX (m/z 1061) (Daiguji *et al.*, 1998) and cooliatoxin (m/z 1061) reported for *C. monotis* (Holmes et al., 1995). An additional LC-MS2 fragmentation was performed (Fig. 7c). Again the daughter ions scheme was not consistent with the characteristic fragmentation of the desulfoYTX side chain (Fig. 7b), and the ion at m/z 1067 was rejected as an YTX analog.

In addition, an extract of VGO780 was injected i.p. into mice, but signs of intoxication were not observed (Cacho, pers. comm.)

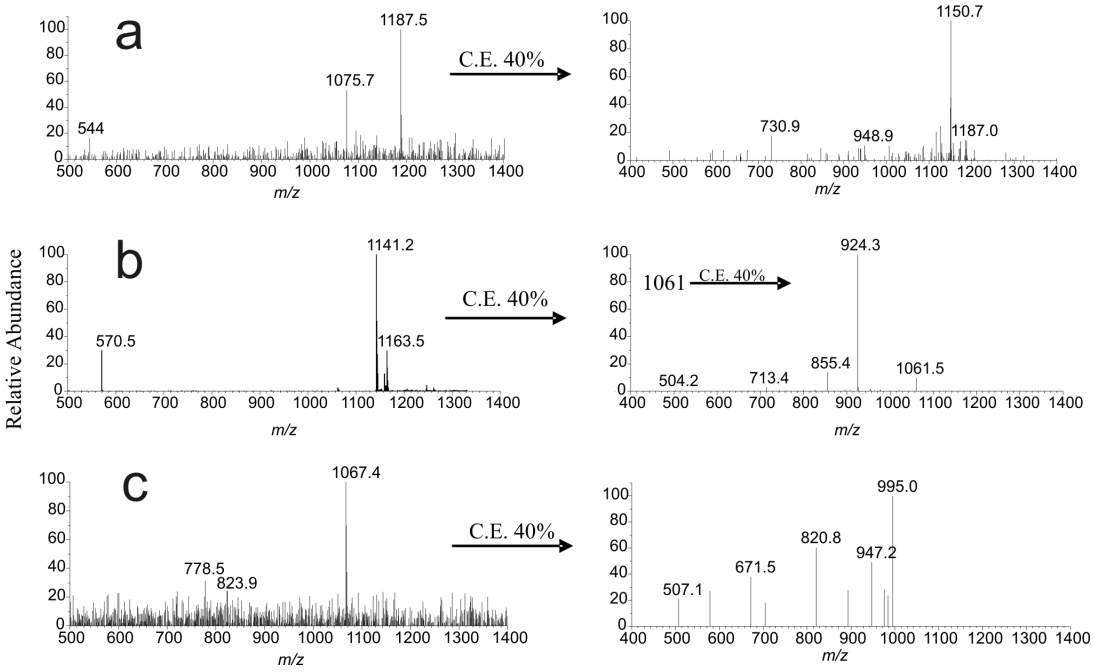

Fig. 7. LC-MS spectra in negative ion mode of more relevant *m/z* ion (left) and MS/MS fragmentation applying a C.E. of 40 % (right): (a) *C. canariensis* VGO780; (b) *P. reticulatum* YTX extract; (c) *C. monotis* CM6V.

DISCUSSION

Morphology and genetics

In order to be able to compare this new species with the type species of the genus, *C. monotis* (Meunier, 1919) it is necessary to interpret the nomenclature of the tabulation used in its original description, which is not kofoidian (Kofoid, 1909), even though this species was described ten years after Kofoid had proposed his tabulation system. (Meunier, 1919) considered 3', 4'and 6" as three «*terminal*» plates. Po and 2' were considered as one «*intermédaire*» plate in which a small kidney shaped «*organ*» is observed. 1', 1", 2", 3", 4" and 6" were considered as seven «*peripherique*» as, in his figures, what we consider 3'' appears split into two plates. The two antapical plates were considered as one over which the sulcus is developed. (Lebour, 1925) reinterpreted Meunier's figures using the Kofoidian tabulation system. Therefore she considered 3 apicals, one of which is pierced by a large apical pore, and was considered as intercalary by Meunier, and 8 precingular plates as she did not realize that 3" was actually split into two plates in the Meunier's drawing. She also considered the possibility that the large 5", which for her was 7", could

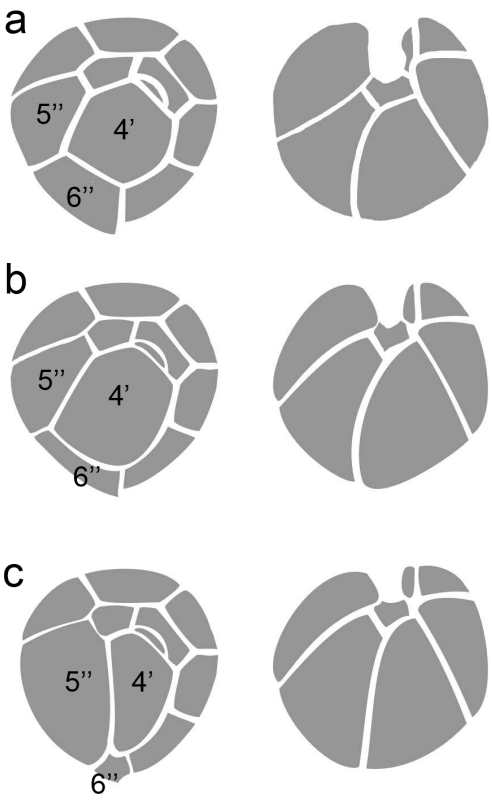

Fig. 8. Ink drawings of *C. canariensis, C. monotis* and C. tropicalis. (a) Epitheca and hypotheca of *C. canariensis*. (b) Epitheca and hypotheca of *C. tropicalis*. (c) Epitheca and hypotheca of *C.monotis* .

be an apical plate as interpreted by Meunier. (Biecheler, 1952) considered 5" as an apical and left open the possibility that 2" together with Po could be both an apical plate or an intercalary plate. As she also considered the plate we consider 6" as being on the left side of the theca, she started to count the precingulars from this plate, so our 1' is for her 2" and our 1'' is for her 2'' and so on. (Balech, 1956) was the first to consider *C. monotis* as having the following plate formula Po, 3', 7", 6c, 5''' and 2'''' which was also followed by (Fukuyo, 1981). Although the latter authors did not mention the error on the Meunier's original description, Meunier had already reported that it is very difficult to examine the dorsal plates of the epitheca so some errors were possible in his description. Based on plate homologies between *Coolia, Ostreopsis* and *Gambierdiscus*, (Besada *et al.*, 1982) interpreted a different plate formula considering 1" as 1' and hence Po, 4', 6", 6c, 5''' and 2"» which is the formula we use in this paper. Once the plate terminology is clear, it is possible to compare *C. canariensis* with the type species of the genus, *C. monotis,* as well as with other more recently described species, *C. tropicalis* and *C. areolata*.

The largest plate of the epitheca of *C. canariensis* is 4' which is centered on the epitheca as in *C. areolata* (Fig. 8a), while in *C. monotis* the biggest plate is 5" (Meunier, 1919, Balech, 1956, Fukuyo, 1981) (Fig. 8c). In apical view, the suture between 4' and 5" in *C. monotis* appears vertical in the middle of the epitheca, (Fig. 8c) while in *C. canariensis* it is clearly displaced to its right side (Fig. 8a) showing 4' as a beret as in *C. areolata*. According to Faust (1995)*C. tropicalis* has a 4' wedge shaped and is displaced to the ventral left side of the epitheca., very different from all the other species of the genus as shown by (Faust, 1995) in her figure 12A. The tabulation pattern of *C. canariensis* is very similar to *C. areolata* but the thecal surface of *C. canariensis* is smooth like in *C. monotis*

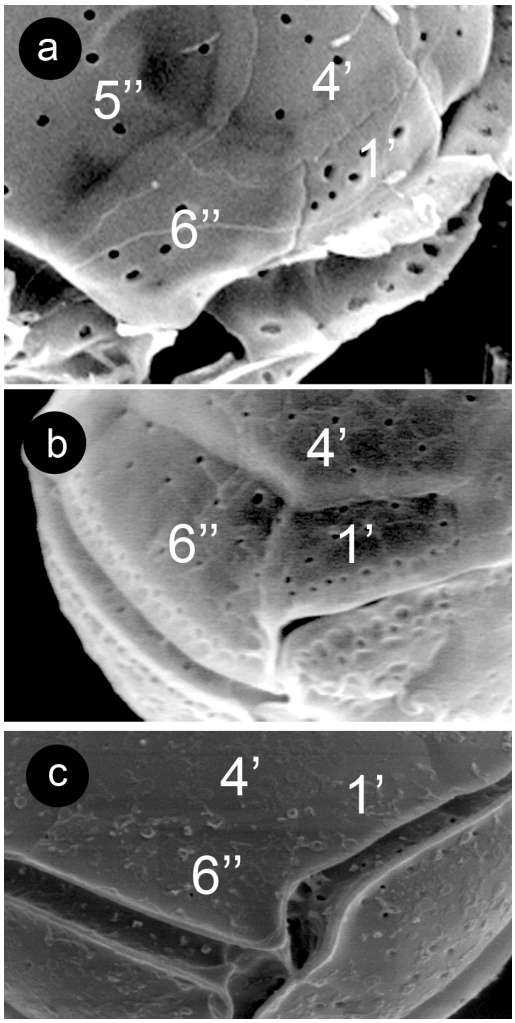

Fig. 9. SEM pictures of details of plates 6" and 1'.(a) *C. monotis*. (b) *C. canariensis*. (c) *C. tropicalis.*

(Faust, 1992, Dolapsakis et al., 2006) and *C. tropicalis* (Faust, 1995) and very different from that of *C. areolata* which is areolated: this being the main distinctive characteristic of that species. Although some ornamentation may be observed in some hypothecal plates of *C. canariensis* (Figs. 2a and 4c), it is very different from the areolation shown by *C. arolata*. (Ten-Hage et al., 2000). Three different types of 6" can be observed in *Coolia* (Figs. 9a and b) and in Fig. 8 of (Faust, 1995): In *C. monotis* it is very small and as wide as it is long (Fig. 9a); in *C. canariensis* and *C. areolata* it is bigger and wider than it is long (Figs. 3a and 9b) (Ten-Hage et al., 2000), and in *C. tropicalis* it is very wide and short (Faust, 1995). The width / length relations of plate 6'' are approximately 1 for *C. monotis*, 2 for *C. canariensis* and *C. areolata,* and 4 for *C. tropicalis*. No significant differences were observed on the tabulation of hypotheca (Fig. 8b and d).

The D1/D2 regions of the LSU ribosomal gene amplified using the oligonucleotide primers of (Scholin et al., 1994) turned out to be useful in delineating the phylogenetic relationships between the different *Coolia* isolates, distinguishing three genetically distinct lineages that were *C. monotis*, *C. canariensis* and a clade including a *C. monotis* from Belize and a *C. malayensis* from Indonesia. In the phylogenetic tree, the clade of *C. canariensis* appeared clearly differentiated with respect to the other clades of *Coolia* species thus supporting the morphological differences found in *C. canariensis* for it to be considered as a new species. It is also different from the other clade comprising the isolates from Belize and Indonesia which probably corresponds to *C. tropicalis*. Within the *C. monotis* clade the European group and a sister group appeared to be well separated phylogenetically. The later group includes isolates from New Zealand and Florida together with a strain from Malaisia deposited in the GenBank under the name of *C. malayense*, which was recently described as *C. malayensis*. This suggests that these other two groups

constitute two species of *Coolia,* being separated by the European *C. monotis* clade, which also comprises the strain RIKZ4 that was isolated very close to the type locality of *C. monotis*. However, no genetic data of *C. tropicalis* and *C. areolata* are available yet to further clarify phylogenetic relationships among species within this genus.

In relation to other genera, (Fensome et al., 1993) included genus *Coolia* in Subfamily Gambierdiscoidea based on the position of the sulcal posterior plate, which on the Taylor-Evitt tabulation model is called posterior sulcal homologue (Z), external to the sulcus. Nevertheless, we demonstrate that this plate is well inside the sulcus (Figs. 2c and 4b), so the taxonomic position of *Coolia* together with *Gambiersiscus* based on this characteristic is not correct. Plates 3' and 2" are never in contact in any species of *Coolia* and they are separated by 2'and 4", while they may be in contact in *Ostreopsis* species. Another important characteristic that differentiates *Coolia* and *Ostreopsis* is the shape of the nucleus, which is U shaped in *Coolia* (Fig. 4e) (Biecheler, 1952, Faust, 1992) as in the genus *Alexandrium* while it is rounded in *Ostreopsis* (Faust & Morton, 1995). In addition, some species of *Ostreopsis* produce palytoxin analogs which have not yet been found in any species of *Coolia* (Riobó et al., 2004, Penna et al., 2005).

Pigments

Members of the Gonyaulacales are typical peridinin-containing dinoflagellates. *Coolia canariensis* shares a similar pigment pattern to *C. monotis* (strains RIKZ4 and SZN43) and *O. ovata* (strain OSO1BR) (Zapata et al. unpublished data) except for the occurrence of the unknown carotenoid 448, which was only detected in *C. canariensis*. (strains VGO775 and VGO787). However, the value of this small carotenoid as a marker pigment will require its chemical characterisation and a more intensive species sampling to evaluate its specificity.

Toxins

LC-MS analysis of the strains studied did not show known characteristic ions for YTXs, but some of the strains showed ions at m/z 1187 and 1067 with mass similar to that of some YTX analogs, but after complementary mass fragmentation the molecules did not give the characteristic fragmentation pattern of YTXs. The loss of water molecules was reported in the MS fragmentation of the cooliatoxin (m/z 1061) described by Holmes et al. (1995) but this, as in the strains studied here, did not produce the fragments at m/z 925 and 855, which are characteristic for yessotoxins (Ciminiello et al., 2000, Ciminiello et al., 2003, Miles *et al.*, 2005).Therefore LC-FLD and LC-MS study revealed that none of the analysed strains of *C. canariensis* and *C. monotis* produced YTX or known YTX analogs.

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REDESCRIPTION OF *C. TROPICALIS* FAUST

INTRODUCTION

When describing *C. tropicalis*, Faust (1995) separated it from *C. monotis* by the length of the apical pore (Po), given as 12 µm long in *C. monotis* versus 7 µm in *C. tropicalis*, the shape of the fourth apical plate, 4' (as 1') oblong and narrow in *C. monotis*, wedge-shaped in *C. tropicalis*, and the shape of the third apical plate 3' (quadrangular in *C. monotis*; pentagonal in *C. tropicalis*). However, the size of Po provided by Faust (1992) is not in agreement with the size obtained from her Figures 2 and 6 (Fig.10), which is only half as long (Aligizaki & Nikolaidis, 2006).

The original description of *C. tropicalis* (Faust, 1995) was based only on SEM images as no LM is referred in the Material and Methods section. The drawings of this species (Fig. 11) given by the author has to be obtained from SEM images, and we can presume that the best of them are those pubulised. The characteristic shape of plate 4' described by Faust (1995) in her drawing cannot be obtained from the published SEM pictures as there is not any complete view of this plate as in her Figure 7 only a small part of the dorsal side of the plate can be obliquely observed, and in Figure 8, most of that plate is covered by detritus. Nevertheless, plate 6'' (labeled by Faust as 7'') can be clearly observed in her Figure 8. We have observed that the shape of this plate is a very important character to distinguish Coolia species. When cells haviing plate 6'' with this shape were observed, the shape of plate 4' was very different form the description given by Faust (1995)

The finding of cooliatoxin in *C. monotis* from Australia (Holmes *et al.*, 1995) triggered many studies elsewhere, but cooliatoxin or similar toxins have not been reported since then. However, *C. monotis* from New Zealand was found to be toxic to mice, causing positive sodium channel activity in neuroblastoma assays (Rhodes *et al.*, 2000). Based on morphology, it is now clear that the species originally reported to produce cooliatoxin was not *C. monotis* (Holmes et al., 1995), but more likely the subsequently described species *C. tropicalis*. However, the mis-interpretation of the shape of plate 4' (labeled as 1') between the scanning micrographs and the drawing of *C. tropicalis* in the original description has been a source of confusion. Recently *C. malayensis* was described from Malaysia, and this species is considered to be the smallest known species of *Coolia* (Leaw et al. 2010). *C. malayensis* is very similar to *C. monotis*, and characters used to differentiate *C. malayensis* are cell size, the shape of the third apical plate, pores in the plates having minute perforations, the length of the pore plate Po, the size of postcingular plate 32 2 2 , the secondary structure of ITS2 (Leaw et al., 2010), and the length-to-width ratio of the cell. The toxicity of this species is unknown.

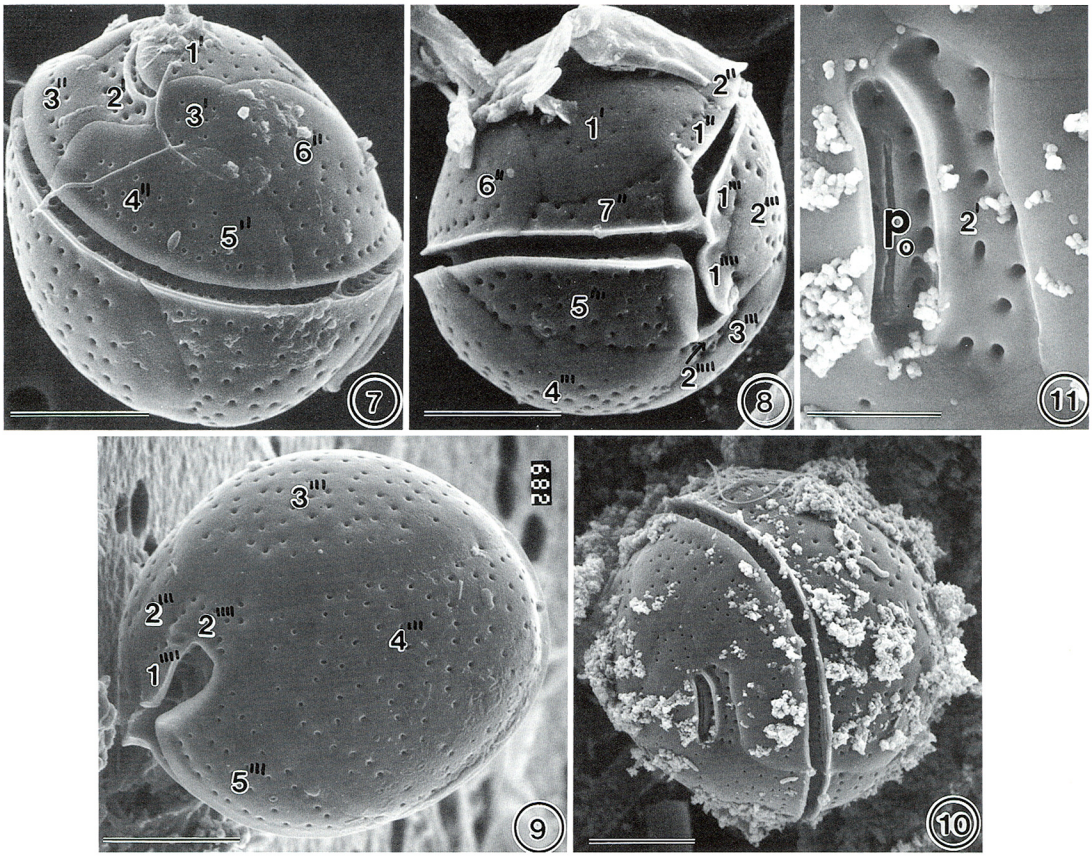

Fig. 10. *Coolia tropicalis*. Original SEM figures from Faust (1995). Fig. 7 is an «*oblique dorsal view of* C. tropicalis *showing the apical pore and the equatorially located lipped cingulum*». Fig. 8. «*Cell in equatorial view*». «*Detritus adheres to the epitheca.*»

C. tropicalis cultures from Malaysia, Indonesia, Australia and Belize, and *C. malayensis* cultures from Malaysia and the Caribbean Sea were acquired or established during the present study. The plates of *C. tropicalis* were examined based on epifluorescence microscopy and scanning electron microscopy (SEM), and molecular sequencing (LSU rDNA) was done to emend the original description of the species. This study aims to clarify the morphology of *C. tropicalis*.

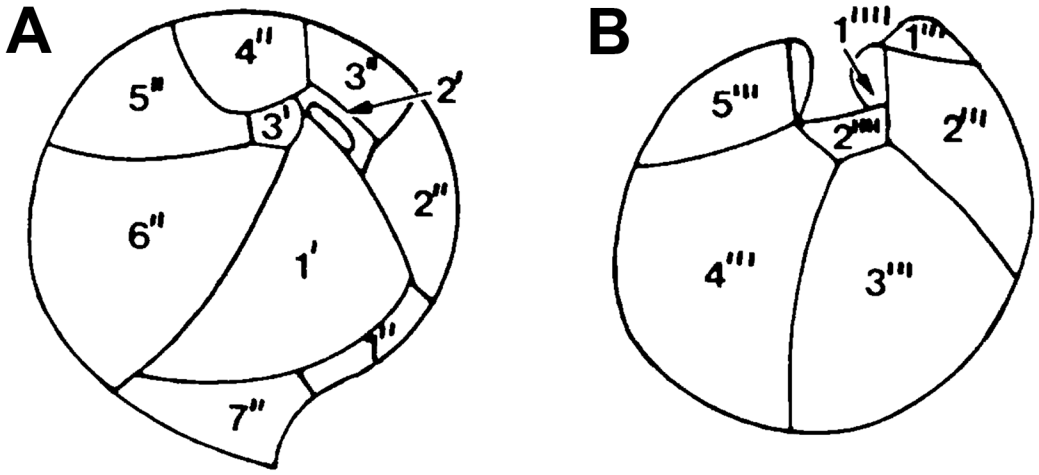

Fig. 11. *Coolia tropicalis*. Original drawings from Faust (1995). «*A) Apical view of epitheca and B) antapical view of hypotheca*».

RESULTS

Redescription of C. tropicalis *Faust 1995*

(Figs. 12, a–d; 13, a–d).

Cells subspherical in ventral view, with numerous golden-brown chloroplasts). Cultured cells from Indonesia 36–37 µm long and 30–35 µm µm wide (n = 3), from Belize 36– 42 µm long and 34–37 µm wide (n = 2; CCMP1744), and the Australian strain 35–47 µm long and 30–45 µm wide (n = 7). Plate formula Po, 4', 0a, 6'',6c, ?s, 5''', 0p, 2''''. Cell surface perforated by scattered pores (Figs. 12, a–d). The Po is situated in the left dorsal side of the epicone, in contact with plates 2', 3', and 4' (Fig. 12d). It was 7.2–7.9 µm long (n = 5) in Malaysian cells, 7.4–8.1 µm long (n =in Belize cells and 9.2–12 µm long (n = 2) in the Australian cells (total mean Po: 8.2 _ 0.76 µm long).

The Po was curved, with a single slit surrounded by pores. The fourth apical plate (4') was the largest on the epicone, but only slightly larger than the precingular plate 5'' (Figs. 12, a, c and d; 13b). The lateral sides of 4' were almost parallel but widened toward the ventral side (Figs. 12, a, c, d; 13, a and b). The apical plate 3' was in contact with Po, 2' , 3'', 4'', 5'', and 4' (Fig. 13b, c). The apical plate 1' (which does not contact the Po plate) and the adjoining precingular plate 6'' were both narrow (Figs. 12, a, c and d; 13b). The 1' plate was about as long as wide, whereas the 6'' plate was ~3x wider than long. In

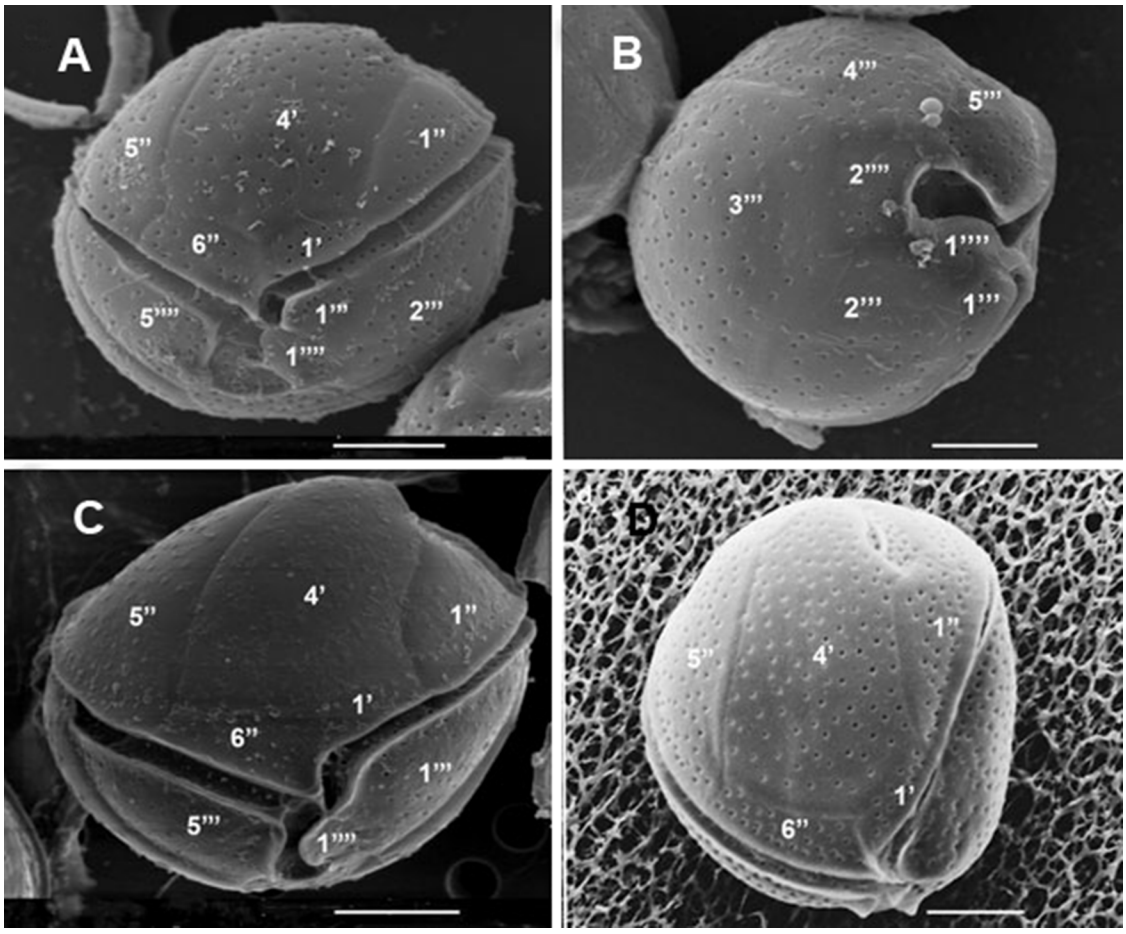

FIG. 12. *Coolia tropicalis*. (a) and (b) Ventral view of epicone and hypocone, respectively of C. tropicalis from Belize,(c) from Indonesia and (d) from Australia. Scale bar = 10 lm.

the hypocone, the plate arrangement fitted with the original description. The postcingular plates 3''' and 4''' were large and occupied most of the hypocone (Figs. 12b; 13, c and d). The two antapical plates 1'''' and 2'''' plates were small and in contact with the sulcal area (Figs. 12b; 13d).

Phylogenetic analyses of Coolia.

In the LSU rDNA phylogenetic analyses *Coolia* fell into three distinct lineages (Fig. 14). The first lineage included *C. tropicalis* strains from Malaysia, Indonesia, and Belize (Fig. 14). The second lineage comprised *C. canariensis* strains from Canary Islands and

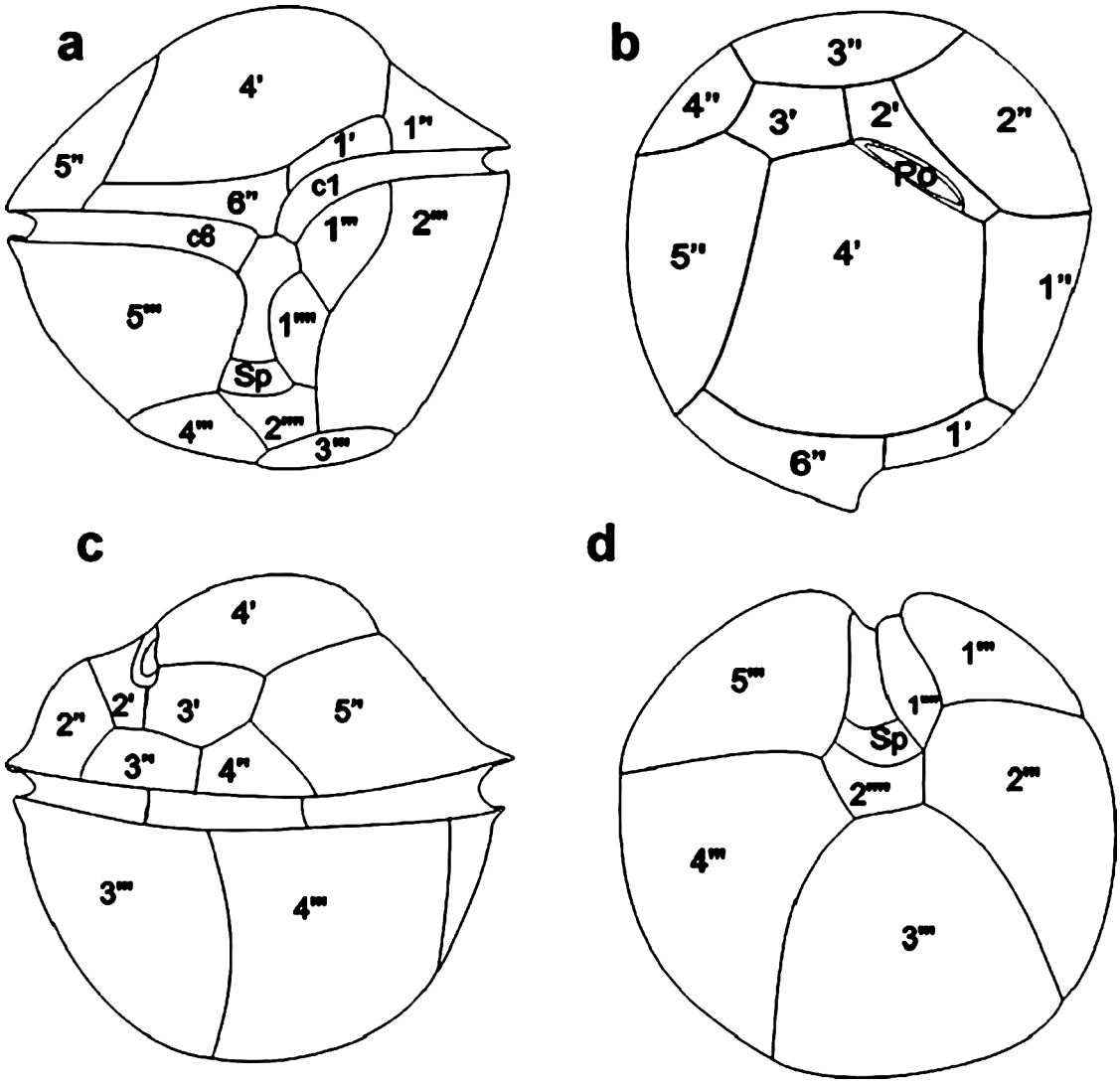

FIG. 13. Ink drawing of *Coolia tropicalis*. (a) Ventral view (b) Apical view (c) Dorsal view (d) Antapical view.

Bay of Biscay (the latter labeled *C. monotis* in Genbank but identified as *C. canariensis* by Laza-Martinez et al. 2011). It appeared as a sister clade to *C. tropicalis*. In the second lineage, the *C. canariensis* strains clustered in two clearly separate clades. The third lineage comprised two very closely related sister groups, (i) a group comprising *C. monotis* strains from Europe (Greece, Spain, Italy, the Netherlands) including strain RIKZ4 isolated 100 km from the *C. monotis* type locality, and (ii) a group representing strains of *C. malayensis* from Malaysia, New Zealand and the Caribbean Sea (Note: the isolates from

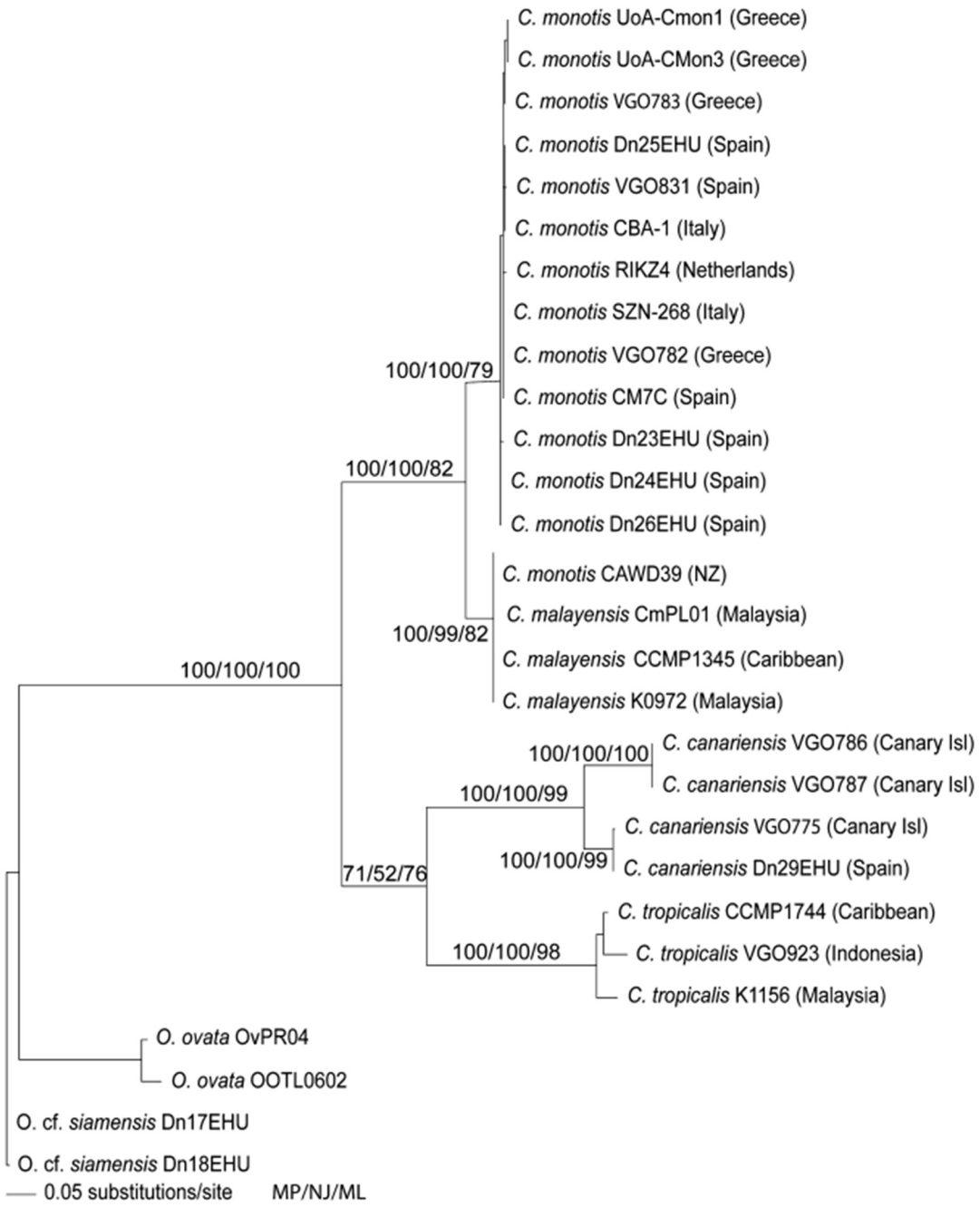

FIG. 14. *Coolia* phylogeny based on maximum likelihood analysis of the D1-D3 region of LSU rDNA. The bootstrap support values shown from left to right on each branch represent maximum parsimony/neighbor joining/maximum likelihood values, respectively.

the latter two localities are listed in Genbank as *C. monotis*). All lineages were well supported by high bootstrap values.

DISCUSSION

Differences from the original description of C. tropicalis.

The morphology of *C. tropicalis* from Malaysia, Indonesia, Belize, and Australia generally fitted the original description from Belize by Faust (1995), with the exception that our cells were ovoid-subspherical in shape compared to spherical in the original description. The size of *C. tropicalis* isolated from the four areas, including the type locality, were slightly larger than in the original description, in which Faust (1995) reported *C. tropicalis* cells to range from 23 to 40 µm long and 25 to 39 µm wide. In this study, the cell length ranged from 35 to 47 µm (40 ± 4.7) and the width from 30 to 45 µm (37 ± 5.2). The larger cells in our cultures compared to the field samples used by Faust (1995) may be the result of culture conditions or the possible presence of planozygotes. The apical pore of *C. tropicalis* was longer than in the original description, varying between 7.2 and 12 µm, compared to 7 µm in Faust (1995). However, only 11 apical pores have been measured and therefore the apical pore range needs to be confirmed by measuring more cells. The main difference from the original description and the main cause of this emendation is the shape of the fourth apical plate. The sutures in the original line drawings showed an angle of about 65° between the 42 plate and the 12 2 and 52 2 plates when cells are seen in apical view. As a result, the plate was described as wedge shaped. In contrast, the *C. tropicalis* cells examined in this study showed the sutures formed by the juncture of the 42 and 12 2 and the 42 and 52 2 plates ran nearly parallel to one another with a slight widening toward the ventral side of the plate. In ventral view, the general shape of *C. tropicalis* observed in this study resembled *C. areolata* and *C. canariensis* but it was narrower than in these species. Scrutiny of the scanning micrographs published with the original description (fig. 8) showed the fourth apical plate of our material and the Belizean material to be similar, and none of our material had a wedge-shaped plate 42 , although the drawing by Faust (fig. 12A) shows a wedge-shaped shape of the fourth apical plate. The fourth apical plate was apparently misinterpreted due to the cell in Faust's figure 8 being obscured by detritus. The third apical plate, 32 , was 5-sided in our material and contacted plates Po, 22 , 42 , 32 2 , 42 2 , and 52 2 . In the original description of *C. tropicalis* (fig. 12A), plate 32 is surrounded by five plates, although it was described as quadrangular. The holotype proposed by Faust (1995) can be maintained, as the error was in the ink drawing describing the species and not the image illustrating the type (fig. 7). Figure 2d from this study is proposed as epitype.

***Morphological differences between* C. tropicalis *and other species of* Coolia.**

In order to differentiate *C. tropicalis* from other *Coolia* species, we compared the Malaysian, Belize, Australian, and Indonesian C. tropicalis with other descriptions of Coolia species (Table 2). *C. tropicalis* differs particularly in size and shape of plate 42 , which is proportionately larger than in the other Coolia species (Table 2). The fourth apical plate of *C. tropicalis* is widest toward the ventral side and situated at the cell center. *Coolia monotis* and *C. malayensis* both possess a narrow fourth apical plate located on the left side of the cell and a large 52 2 (Table 2). The fourth apical plate of *C. canariensis*, *C. areolata*, and *C. tropicalis* is positioned in the center of the epicone (Table 2). The hypocone is more or less similar in all species, plate 32 2 2 being the largest plate in the hypocone (Meunier 1919, Balech 1956, Ten-Hage et al. 2000, Fraga et al. 2008, Leaw et al. 2010). The drawings of Meunier (1919) and Balech (1956) also show plate 32 2 2 of *C. monotis* to be the largest postcingular plate. Cooliatoxin was described to be produced by *C. monotis*, as the genus Coolia was assumed to be monotypic at the time (Holmes et al. 1995, Fig 1). Both reexamination of the 62 2 and 42 plates (in this study) and the published micrographs and drawings indicate that the toxic clone was *C. tropicalis* (Holmes et al. 1995).

Phylogenetic analysis of* Coolia *species.

The phylogenetic analyses showed *C. tropicalis* to be clearly separate from *C. canariensis*, *C. malayensis* and *C. monotis*, with high bootstrap values. This agrees with the suggestion by Fraga et al. (2008), that *C. tropicalis* is a separate taxon. *Coolia* strains isolated from Belize and Indonesia appeared in the same group as *C. tropicalis* from Malaysia. Morphologically, the fourth apical plate of *C. tropicalis* from South East Asia differed in the shape and size from the original description, as it was larger than cells described from Belize by Faust (1995). *C. malayensis* in our study grouped with the other strain of *C. malayensis* (CmPL01) from Malaysia and formed a sister group to *C. monotis* supporting Leaw et al. (2010) who used morphology, phylogeny and ITS rRNA structure to separate *C. malayensis* from *C. monotis*. All *C. monotis* strains from European countries group in the same lineage. A phylogenetic study by Penna et al. (2005), using 5.8S rDNA ITS, showed *C. monotis* grouping into two clades (i) a Mediterranean and northeast Atlantic clade, and (ii) an Asian/Florida clade. But the latter clade corresponds to what is now termed *C. malayensis*. Two clades of *C. canariensis* appear in the tree and deserve further study to clarify whether they present different species.

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CAPÍTULO 3

GÉNERO *GAMBIERDISCUS*

GAMBIERDISCUS EXCENTRICUS SP. NOV (DINOPHYCEAE), A BENTHIC TOXIC DINOFLAGELLATE FROM THE CANARY ISLANDS (NE ATLANTIC OCEAN)

ABSTRACT

A new benthic toxic dinoflagellate is described from the Canary Islands, Spain. *Gambierdiscus excentricus* sp. nov. was isolated from seaweeds growing in tidal ponds and was observed in winter and summer. Its morphology was studied by means of Light Microscopy (LM) and Scanning Electron Microscopy (SEM); *G. excentricus* is a lenticular species having a Po plate ventrally displaced in relation to other species of the genus *Gambierdiscus*. Phylogenetic trees from large subunit (LSU) of ribosomal RNA gene sequences displayed a topology confirming that *G. excentricus* clustered in its' own group, separated from the rest of *Gambierdiscus* species and with *G. australes* as its closest relative. Pigment composition studied from *G. excentricus* cultures, included peridinin, as the major carotenoid, chlorophyll *a* and the accessory chlorophylls c_1 and c_2. The Neuroblastoma cell-based assays for ciguatoxins (CTX) and maitotoxin (MTX) confirmed *G. excentricus* as a CTX- and MTX-like compounds producer. The finding of a toxic species of *Gambierdiscus* in the Canary Islands may explain the recent reported cases of ciguatera in the area.

INTRODUCTION

Ciguatera fish poisoning (CFP) is a food-borne disease widespread in tropical and sub-tropical marine areas affecting mainly the Caribbean Sea, Polynesia and other areas in the Pacific, Indian Ocean (Lewis, 2006) although it has been also recently reported in the Canary Islands (Spain), a temperate area (Pérez-Arellano et al., 2005) and in Madeira (Gouveia et al., 2010; Otero et al., 2010) . CFP occurs after consumption of fish contaminated with ciguatoxins (CTXs) (Alfonso et al., 2005) but presence of additional toxins has been also proposed and cannot be discarded (Anderson and Lobel, 1987). Marine benthic dinoflagellate of the genus *Gambierdiscus* Adachi et Fukuyo (Adachi and Fukuyo, 1979; Yasumoto et al., 1977) are responsible for the production of CTXs further transmitted through the food web among reef fishes (Alfonso et al., 2005). The same genus may also produce other toxins i.e maitotoxins (MTXs), gambierol and gambieric acid. MTXs have been found in the viscera of herbivorous fish but are unlikely

to produce human illness due to their low capacity for bioaccumulation in fish tissue and low oral potency (Alfonso et al., 2005).

The genus *Gambierdiscus* had been considered monospecific for fifteen years with *Gambierdiscus toxicus* Adachi & Fukuyo (Adachi and Fukuyo, 1979) as the only described species, a thecate gonyaulacoid dinoflagellate anteroposteriorly compressed with lenticular shape. The original plate formula was defined as Po, 3', 0a, 7'', 6c, 8s, 6''', 1p, 1'''' (Adachi and Fukuyo, 1979). *G. belizeanus* Faust (Faust, 1995) was the second species of the genus and it is easily distinguished from *G. toxicus* in having an ornamented theca and some differences in relation to the shapes of plates. The third species being described was *G. yasumotoi* Holmes (Holmes, 1998), a species very different from the other in being globular instead of discoid. Later, the diversity of the genus was found to be much higher than expected and recently seven new species have been added to the genus (Chinain et al., 1999; Litaker et al., 2009) based on morphology and on genetics which helped to find semicryptic species (Litaker et al., 2009; Richlen et al., 2008). Genetic sequences enabled even to find that the original description of *G. toxicus* was based on more than one species making it necessary to describe a new epitype of the species (Litaker et al., 2009).

The Canary Islands archipelago (Fig. 1) is bathed by the Canary Current which is the eastern boundary current of the subtropical North Atlantic gyre. The area is characterized by low biomass and very oligotrophic waters where nutrients are depleted in summer (Cianca et al., 2007; Neuer et al., 2007). In this paper we describe *Gambierdiscus excentricus*, a new toxic dinoflagellate found in the Canary Islands coasts and report the presence of *Gambierdiscus* cf. *polynesiensis* in the same area. In addition to the taxonomic description of *G. excentricus*, production of toxins was examined.

MATERIALS AND METHODS

Source of specimens and culture conditions

Samples were collected at several locations in the Canary Islands' archipelago in the NE Atlantic Ocean (Fig. 1): 1) Punta Hidalgo, a rocky shore on the north coast of Tenerife, (28º 34' N, 16º 19' W) on March 28th, 2004; 2) Charca del Conde, La Gomera (28º 05' N, 17º 20' W) on November 15, 2005; and 3) Playa Las Cabras, La Palma (28º 29' N, 17º 49' W) on March 13, 2010. Samples of small mixed seaweeds and turf in grooves were collected from tidal pools on the rocks during low tide or from drifting seaweeds very near the coast and placed in plastic bottles and shaken. Afterwards, the gross particles were removed and the remaining seawater was used for cell isolation. Isolation was carried out by a capillary pipette with the aid of a ZEISS Invertoscop D microscope (Carl Zeiss

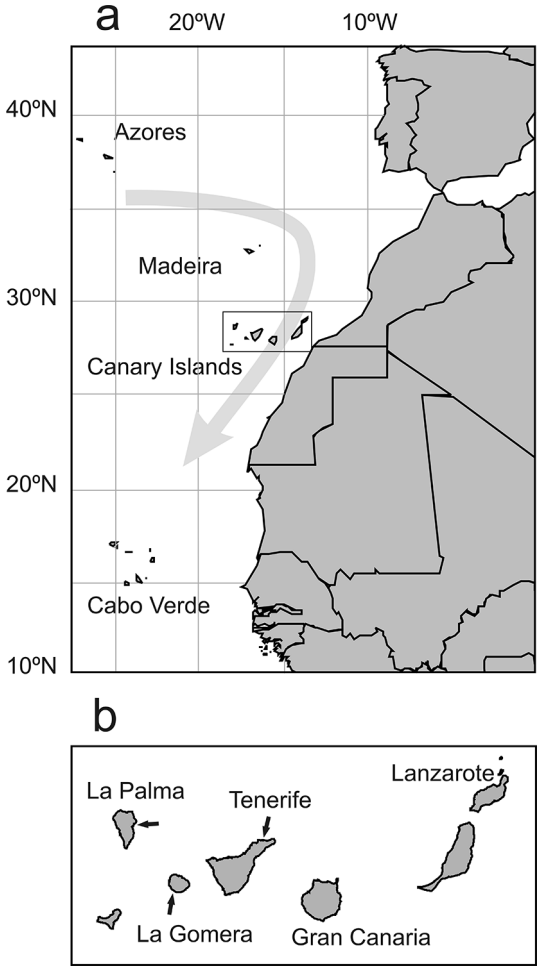

Fig. 1. (a) Map of the East Atlantic archipelagos. (b) Map of the Canary Islands showing the localities where *Gambierdiscus excentricus* was found.

AG, Germany). Isolated cells were incubated in 96 microwells plates in half strength K medium without silicates (Keller et al., 1987) made with seawater from Ría de Vigo (NW Spain) with a salinity adjusted to 34 psu and incubated at 25 ºC and a photon irradiance of about 90 µmol m^{-2}'»s^{-1} of PAR measured with a QSL-100 irradiameter (Biospherical Instruments Inc. San Diego, CA, USA) and at a 14:10 L:D photoperiod. The cultures were transferred to 100 mL Erlenmeyer flasks and to 50 mL polystyrene tissue culture flasks. The cultured strains VGO790, VGO791 and VGO792 were from Tenerife Island and VGO1035 from La Palma Island and all were deposited at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo.

Light microscopy

Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence with an UV lamp. The cultured cells were observed alive or fixed with formalin. For plate pattern identification the cells were stained with Fluorescent Brightener 28 (Sigma-Aldrich, St Louis, MO, USA) following a modified technique (Fritz and Triemer, 1985). Other cells were dissected, squashing the cells by gently pressing the cover slip over them occasionally with the aid of sodium hypochlorite. Microphotographs were taken with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera. When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and were then merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). Cell size was measured by bright field LM on living cells on calibrated digital photographs. Cells stained with Fluorescent Brightener 28 (Sigma-Aldrich, St Louis, MO, USA) were also observed with a Leica

TCS SP5 confocal microscope with UV light (Leica Microsystems GmbH, Wetzlar, Germany) at the CACTI facilities (Universidade de Vigo, Spain). The nucleus was stained using SYBR Green (Molecular Probes, Eugene, OR, USA) following a modified method (Figueroa et al., 2010) as follows: A 10 mL aliquot of culture was fixed with 0.5% paraformaldehyde for 10 min and washed in PBS pH7.0 (Sigma-Aldrich, St.Louis, USA) by centrifugation at 1200g during 10 min. Chlorophyll was extracted by resuspending the pellet in 5 mL of cold methanol and then storing the suspension overnight in the refrigerator. The cells were then washed twice in PBS (pH 7.0) as described above and the pellet was stained with a 1:200 solution of SYBR green in PBS 0.01M (pH7.4) and observed in a Leica DM LA epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) with blue excitation and photographed with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera. The autofluorescence of the chloroplasts was photographed with a Canon EOS 5D Mark II (Canon Inc., Tokyo, Japan) digital camera.

Scanning Electron Microscopy

Five mL of exponentially growing cultures were fixed with glutaraldehide (GTA) at a final concentration of 4%. After two hours at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH and 100% hexamethyldisilazane (HMDS). After being air dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a Phillips XL30 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

Nomenclature

In this study, a modified Kofoid tabulation system (Kofoid, 1909) as described in (Besada et al., 1982) was followed to name the plates therefore allowing comparisons with other genera. The main differences are: In the epitheca, we considered as the first apical plate (1') what most of the authors consider as first precingular plate (1'') and in the hypotheca, second antapical plate (2'''') instead of 1p, and sulcal posterior (S.p.) instead of second antapical (2'''') (More details in section 4.1.). The terms «length» as apical/antapical distance, «width» as transdiameter and «depth» as dorso/ventral distance were used for the cells dimensions.

DNA extraction

Single cells of *Gambierdiscus* were picked up with a micropipette, washed in three distilled water droplets, and stored overnight at -80 ºC in 200 µL tubes. Prior to direct PCR on these single cells, samples were heated at 94 ºC during 1 min in the thermal

cycler. DNA extracts were also used for amplification following a Chelex extraction procedure (Richlen and Barber, 2005) from 2-5 cells of *Gambierdiscus*. Single cells were isolated from cultures and washed in sterile dH_20 before being placed in a 200 µL tube containing 10 µL of 10x PCR buffer. The tubes were stored overnight at -80 ºC. Prior to DNA extraction, the tubes were centrifuged to settle the cells and 30 µL of 10% Chelex 100 (Bio-Rad, Hercules,California, USA) in dH_2O was added. The tubes were boiled at 95 ºC in a Eppendorf Mastercycler EP5345 thermocycler (Eppendorf AG, New York, USA) for 10 min, then vortexed. The boiling and vortex steps were done twice and samples were centrifuged (13,000 rpm for 1 min). The supernatants were transferred to clean 200 µL tubes avoiding to carryover the Chelex beads. Samples were stored at -20 ºC until PCR amplification.

PCR amplification and DNA sequencing

The D1-D3 and D8-D10 regions of the LSU gene were amplified using the pairs of primers D1R/LSUB (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3' (Litaker et al., 2003; Scholin et al., 1994), and FD8/RB (5'- GGATTGGCTCTGAGGGTTGGG-3'/5'- GATAGGAAGAGCCGACATCGA-3' (Chinain et al., 1999), respectively, to produce readable sequences ranging 820-900 nucleotides. The amplification reaction mixtures (25 µL) contained 4 mM $MgCl_2$, 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, California, USA), and 2 µL from the single cell Chelex extractions. The DNA was amplified in a Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) following the conditions detailed elsewhere (Chinain et al., 1999; Litaker et al., 2003). A 10 µL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and SYBR Safe DNA gel staining (Invitrogen, California, USA).

The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA). Purified DNA was sequenced using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) and migrated in an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain).

The D1–D3 and D8–D10 sequences obtained in this study were deposited in GenBank (GenBank ID: HQ877874), (GenBank ID: JF303063-GenBank ID: JF303077).

Phylogenetic analyses

LSU sequences were inspected and aligned using CLUSTALW multiple alignment in Bioedit (Hall, 1999). Uncorrected genetic distances (p; number of substitutions per

site) were calculated for the original alignments using DNAdist v3.5c in Bioedit. Poorly aligned positions and divergent regions were checked using the GBLOCKS software (Castresana, 2000). A final number of 363 and 525 bases (38% and 66% of the original positions in D1-D3 and D8-D10, respectively) were saved by GBLOCKS. The final alignments were converted to nexus files using SeqVerter 2.0 (GeneStudio, Inc., USA).

The phylogenetic relationships were determined using a General Time Reversible model (GTR) in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001). The program parameters were statefreqpr = dirichlet (1,1,1,1), nst = 6, rates = invgamma, nswaps = 1. The phylogenetic analyses involved two parallel analyses, each with four chains. In both parallel analyses there was one cold and three incrementally heated chains, where the heat of the ith chain is B =1/[1 + (i 2 1)T] and T=0.02. Starting trees for each chain were selected randomly using the default values for the MrBayes program. The corresponding number of unique site patterns was 189 and 158 in D1-D3 and D8-D10 analyses.

The number of generations used in these analyses was 400,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization (''burn-in'' phase). All final split frequencies were less than 0.012. For comparative purposes, phylogenetic analyses were also conducted for each dataset after estimating different models of DNA substitution and associated parameters with Modeltest 3.7 (Posada and Crandall, 1998). Phylogenetic trees were obtained using a Tamura and Nei (1993) model with ã distribution (TrN + G) in PAUP 4.0b10 (Swofford, 2002) according with Modeltest 3.7 settings. Bootstrap values were estimated from 1000 replicates.

Pigment analyses

Cultures were examined by light microscopy before carrying out HPLC pigment analysis to ensure the cells were healthy and presented good morphology (absence of alterations of the general structure). Cells were harvested 3 hours into the light cycle from cultures in exponential growth phase. Ten mL of culture were filtered onto Whatman GF/F filters (Whatman International Ltd. UK) under light vacuum. Filters were frozen immediately at -25 ºC, and analyzed within 12 hours. Frozen filters were extracted under low light in Teflon-lined screw capped tubes with 5 mL 90% acetone using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 minutes in an ultrasonic bath. Extracts were then filtered through 25 mm diameter syringe filters (MFS HP020, 25 mm, 0.20 µm pore size, hydrophilic PTFE,) to remove cell and filter debris. An aliquot (0.5 mL) of methanol extract was mixed with 0.2 mL of water and 200 µL was injected immediately. This procedure avoids peak distortion of early eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection in an HPLC system (Latasa et al., 2001). Pigments were separated using

a Waters (Waters Corporation, Milford, MA) Alliance HPLC System consisting of a 2695 separations module, a Waters 996 diode-array detector and a Waters 474 scanning fluorescence detector. Pigment separation was performed following previous work (Zapata et al., 2000), with a reformulated mobile phase A described below. The column was a C8 monomeric Waters Symmetry (150 x 4.6 mm, 3.5 µm particle-size, 100 Å pore-size). Eluent A was methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v). Eluent B was methanol: acetonitrile: acetone (20:60:20 v/v/v). Elution gradient was: (time: %B) t0: 0%, t22: 40%, t28: 95%, t37: 95%, t40: 0%. Flow rate 1.0 mL'»min^{-1} and column temperature was 25 ºC. Solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After checking for peak purity, spectral information was compared with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see (Jeffrey and Wright, 1997).

Toxin analysis

Cultures of *G. excentricus* (strains VGO790, VGO791 and VGO792) were transferred to IRTA Laboratory where they were cultured in 1L Fernbach in a 33 practical salinity unit (psu) modified ES medium (Provasoli, 1968) at 24 ºC under a 12:12 light:dark regime with a photons flux rate of 80 µmol photon m^{-2} s^{-1} (QSL-2100 Radiometer, Biospherical instruments, San Diego, USA) and under permanent aeration. When cultures reached its stationary growth phase with cell densities of 1050, 2231 and 1217 cells mL^{-1} for strains VGO790, VGO791 and VGO792 respectively, cells were harvested through filtration using Whatman GF/F filters (Whatman International Ltd. UK). Filters were stored in absolute methanol at -20 ºC until toxin extraction.

For toxin extraction, GF/F filters were sonicated during 30 minutes at 38% amplitude (Sonics Vibracell, Newton, USA) in an extraction volume (Ve) of absolute methanol proportional to total cell density with Ve in mL equivalent to 10 x 10^6 cells. Methanol was further recovered after 5 minutes centrifugation at 4 ºC at 600g (Joan MR23i, Sant Herblain, France). This procedure was repeated one time with absolute methanol and twice with methanol:water (50:50, v:v) with the same Ve. Supernatants were pooled and evaporated until dryness at 40 ºC (Büchi R-200 or Büchi Syncore, Flawil, Switzerland). Extracts were finally dissolved in absolute methanol and kept at -20 ºC until analysis.

The Neuro-2a CBAs specific for CTXs (Manger et al., 1995) and MTXs (Caillaud et al., 2010c) were used for the determination of CTX- and MTX-like toxicity in *G. excentricus* crude extracts. Neuro-2a cells (ATCC, CCL131) were maintained in 10%

foetal bovine serum (FBS) RPMI medium (Sigma, St Louis, MO, USA) at 37 ºC in a 5% CO_2 humid atmosphere (Binder, Tuttlingen, Germany) as previously described in (Cañete and Diogène, 2008). For experiments, cells were inoculated in a 96-well microplate at a density of 35,000 cells per well and incubated 24h before cytotoxicity assays under the same conditions as described for cell maintenance.

In order to specifically detect the presence of CTX-like compounds, Neuro-2a cells were first treated with 0.1 nM ouabain and 0.01 mM veratridine (V) (Sigma-Aldrich, St Louis, MO, USA) previous exposure of Neuro-2a cells to *G. excentricus* crude extracts during 24 hours as described previously (Caillaud et al., 2011; Cañete and Diogène, 2008). Sensitivity of the Neuro-2a cells to the presence of CTX was calibrated using a standard solution of Pacific type 1 CTX (CTX1B) provided by Dr. R.J. Lewis (The Queensland University, Australia).

For the detection of MTX-like compounds, Neuro-2a cells were first treated with 30µM SK&F 96365 (Sigma-Aldrich, St Louis, MO, USA) during 30 minutes previous exposure to *G. excentricus* crude extracts during 2.5 hours as described before (Caillaud et al., 2011; Caillaud et al., 2010c). The Neuro-2a CBA for MTX was calibrated using a MTX standard solution which was a generous gift from Prof. T. Yasumoto (Japan Food Research Laboratory, Japan).

After exposure of Neuro-2a cells to standards or *G. excentricus* crude extracts for the determination of CTX-like and MTX-like toxicity respectively, toxic effects were measured using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT (Sigma-Aldrich, St Louis, MO, USA) cell viability evaluation (Mosmann, 1983) as described in Manger et al. (1993). Absorbance was read at 570 nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA) and absorbance values were expressed in percentage of viability respect to its respective control (with and without O/V or SK&F 96365 treatment).

Results of cell viability were analyzed using the software Prism 4 (GraphPad, San Diego, CA, USA). A dose-response curve fit with sigmoid regression curve (with variable slope) was determined for each experiment and allowed estimating the concentration of *G. excentricus* extract or standards that inhibited 50% cell viability (IC_{50}) for each experimental condition (with and without O/V or SK&F 96365 treatment). IC_{50}s were further used as a toxicological parameter for the qualitative and quantitative estimation of the content of CTX- and MTX- like compounds produced by *G. excentricus*. Significant differences between means of IC_{50}s or toxin contents were analyzed using unpaired t-test (comparison of two means) and ANOVA (comparison of three or more means) with a 95% confidence level.

Production of CTX-like compounds by *G. excentricus* was identified when differences between IC_{50}s for O/V treated and non treated cells were significant ($p < 0.05$). When unspecific toxicity was measured in absence of O/V treatment (non attributable to CTX-like compounds), the content in CTX1B was quantitatively estimated by substituting the quantity of CTX1B responsible for the IC_{50} of the CTX1B calibration curve (with O/V treatment) for the number of *G. excentricus* cells also responsible for the IC_{50} in both experimental conditions (O/V treated and non-treated cells). The equivalent of CTX1B in *G. excentricus* cells was finally estimated after subtraction of the content of CTX1B equivalents estimated with O/V treatment with the content of CTX1B equivalents estimated without O/V treatment as described previously. (Caillaud et al., 2011; Caillaud et al., 2010a; Caillaud et al., 2010b; Lartigue et al., 2009).

When differences between IC_{50}s for SK&F 96365 treated and non treated cells were significant ($p < 0.05$), production of MTX-like compounds by *G. excentricus* was qualitatively determined by the measurement of a dose-ratio (DR) above 1 (Caillaud et al. 2010b). When DR>1, the content in MTX equivalents was quantitatively estimated by substituting the quantity of MTX responsible for the IC_{50} of the MTX calibration curve with SK&F 96365 treatment for the number of *G. excentricus* cells responsible for the IC_{50} of the microalgal extract with SK&F 96365 treatment (Caillaud et al., 2011; Caillaud et al., 2010b).

RESULTS

Gambierdiscus excentricus *S. Fraga sp. nov.*

> *Cellulae photosyntheticae quarum forma lenticularis est et mensurae mediae earum sunt: 97ìm positione dorsiventrali, 83 ìm latitudine et 37 ìm longitudine. Thecae formula est: Po, 4', 0a, 6", 6c, ?s, 5"', 0p, 2"". Thecae laminae sunt laeves et poros rotundos et ovales uniformiter ordinatos habent. Lamina apicalis pori, Po, ovalis est, habet rimulam hamuli forma et ventraliter lapsa. Lamina prima apicalis 1' parva est. Secunda apicalis lamina 2' maior ex epitheca est et suturam 2'/3' habet fere duplicer longiorem quam suturam 2'/4'. Placae 1' et 6" parvissimae sunt et respiciunt ad posteriorem caellulae partem torsionis causa areae flagellaris, quae habet cavum ex quo dua flagella emergunt, quorum longitudinale perpendiculariter projicitur. Lamina S.p. locatur in hypotheca extra sulcum. Lamina 2"" duplo longa est quam lata. Nucleus arcus formam habet et locatur in parte dorsale caellulae et cuspides ejus diriguntur ad ventralem partem. Toxica est et generat ciguatoxina atque maitotoxina.*

Cells of *G. excentricus* are lenticular in shape with average depth 97 ± 8 (84-115) µm, width 83 ± 10 (69-110) µm, and length 37 ± 3 (34-41) µm. Thecal plate formula: Po, 4', 0a, 6'', 6c, ?s, 5''', 0p, 2''''. Thecal plates are smooth with evenly distributed round to oval pores. Apical pore plate Po is oval with a fishhook-shaped slit and is ventrally

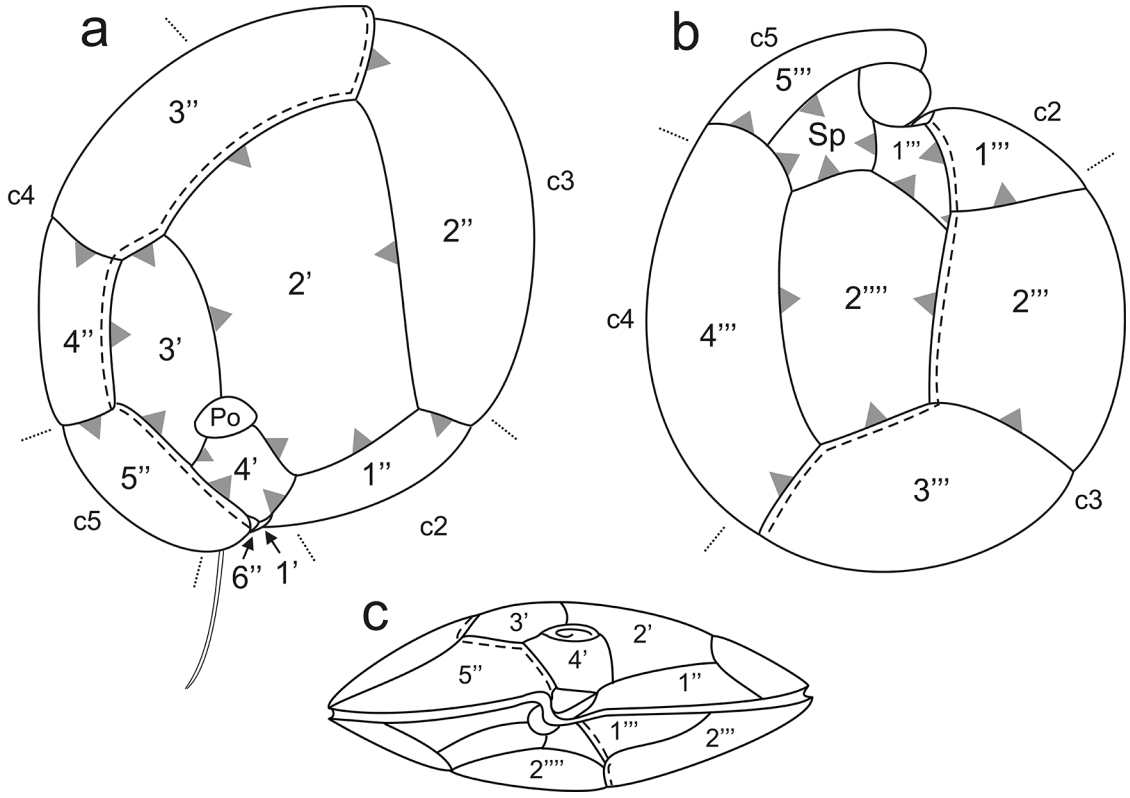

Fig. 2. Ink drawings of *Gambierdiscus excentricus*. (a) Apical view. (b) Antapical view. Gray arrows indicate direction of plates overlap. (c) Ventral view. Dotted lines show fission line.

displaced. First apical plate, 1’ is very small. Second apical plate 2’ is the largest of the epitheca and has the suture 2’/3’ about twice as long as the suture 2’/4’. Plates 1’ and 6” are very small and facing the posterior part of the cell due to the torsion of the flagellar area which forms a hollow from which two flagella emerge, the longitudinal one being perpendicularly projected. S.p. is situated out of the sulcus in the hypotheca. 2’”’ is about twice as long as wide. The nucleus is arc shaped and is located in the dorsal part of the cell with points towards the ventral side of the cell. Cells are photosynthetic.

Holotype: Fig. 2 from clonal strain VGO790, barcoded in GenBank (GenBank ID: JF303074) , (GenBank ID: HQ877874) and (GenBank ID: JF303065) and with preserved DNA at Centro Oceanográfico de Vigo (IEO). Clone VGO790 was collected on March 28th, 2004 as an epiphyte on small filamentous macroalgae and turf on a tidal pond in Punta Hidalgo, Tenerife Island, Spain (Fig. 1). It is deposited at the Culture Collection of Harmful Microalgae of Centro Oceanográfico de Vigo (CCVIEO).

Etymology: Refers to the position of the Po plate which is ventrally displaced compared to other species of *Gambierdiscus* in which it is centrally located.

Type locality: Punta Hidalgo, Tenerife Island (Spain) (28º 34' 37"N; 16º 19' 42"W) (Fig. 1).

Distribution: G. excentricus is only known from the Canary Islands of Tenerife, La Gomera and La Palma.

Morphology

Armored lenticular cells, anteroposteriorly compressed, with average depth (dorso-ventral axis) 97 ± 8 (84-115) µm, width 83 ± 10 (69-110) µm, and length (Antero - posterior axis) 37 ± 3 (34-41) µm. In apical or antapical view the cell is oval and indented in the ventral area showing a lobe in the right side (Figs. 2, 3, 4). In recently divided cells this lobe is more prominent in one of the two daughter cells (Fig. 5). Young cells are oval in apical view, but the dorsal side of old cells is flat (Fig. 5b). Epitheca and hypotheca are similar in height, smooth and covered by evenly distributed round pores of about 0.5 µm in diameter and at a concentration of 54 ± 10 per 100 μm^{-2} (Fig. 4). The plate formula is Po, 4', 0a, 6'', 6c, ?s, 5''', 0p, 2''''. Po is ventrally displaced and has a fishhook-shaped slit surrounded by a row or pores (Fig. 4d). It contacts three apical plates: 2', 3' and 4' which

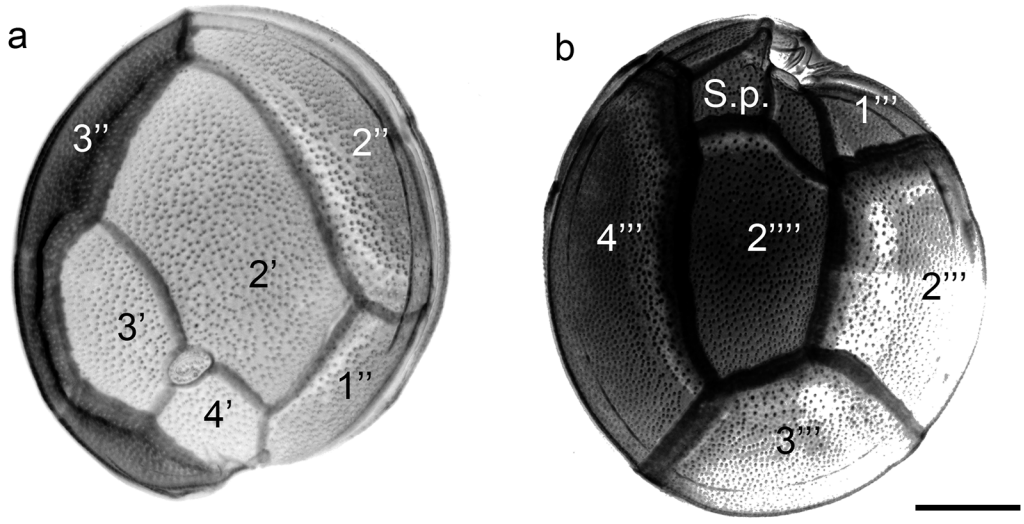

Fig. 3. Confocal microscope image of *Gambierdiscus excentricus* after calcofluor staining. (a) Apical view. (b) Antapical view. Scale bar. 20 µm.

overlapped it (Fig. 2). Plate 1' is very small and arrow point shaped; it does not contact Po but contacts 4' with the anterior point and is compressed by 1'' and 6'' forming like a groove with small wings having a cingulum-like appearance (Fig. 4c). The tiny 1' and 6'' are orientated towards the posterior side of the cell so they are not visible in apical view and only the lists bordering 1' are visible in this view. Plate 2' is more or less rectangular and is the biggest of the epitheca; it is dorsally pointed and it is overlapped by 3', 4', 1'', 2'' and 3''; as a result of the ventrally displacement of Po, its 2'/3' suture length is more than twice as long as 2'/4' suture length (Figs. 2a, 3a, 4a, 6). Plate 3' is dorsoventrally elongated and overlaps 2', 4' while it is overlapped by 3'', 4'' and 5''. Plate 4' is smaller than 2' and 3', and in the ventral end overlaps the tiny 1' and 6'' plates. Plate 1'' is five sided and overlaps contacts 1', 4', and 2', and it is overlapped by 2''. Plate 2'' is four sided and together with 3'', which is five sided, they are the biggest of the precingular series and occupy the whole dorsal part of that series. Plate 2'' overlaps 1'' and 2', and is overlapped by 3''. Plates 1'', 4'' and 5'' have an intermediate size and plate 6'' is very small. The cingulum is descendent one girdle width but, in ventral view the flagellar area appears twisted clockwise giving the appearance of being ascendant (Fig. 4c). It is composed of 6 plates being c1 and c6 curved due to the torsion of the flagellar area. The sulcus forms a hollow and S.p. is out of it forming part of the hypotheca. S.a. is in contact with 1' and 6''. The hollow is limited in the posterior side by the anterior edges of 5''', S.p., and 1'''. It was not possible to analyze all the sulcal plates. The longitudinal flagellum emerges in the equatorial plane perpendicularly from the hollow and below plate 5'' when observed in apical view (Fig. 2a). The transverse flagellum finished well inside the hollow.

The hypotheca is composed by five postcingular plates and two antapical plates in addition to S.p. which being out of the sulcus is considered as 2'''' by many authors. 1''' is triangular and is the smallest of the series, 2''' trapezoidal being the dorsal part wider than the ventral part. Plate 3''' is four sided and dorsally placed. Plate 4''' is elongated and occupies most of the right side of the postcingular area being the biggest of the postcingular series. Plate 5''' is small and twisted. In the antapical series, 1'''' is more or less symmetrical to S.p. and contacts 1''', 2''', 2'''' and S.p. (Figs. 2b, 3b, 4c). Plate 2'''' contacts five plates, 1'''', 2''', 3''', 4''' and S.p. and it doesn't contact 5'''. The width of 2'''' is about one third of the transdiameter and is about twice as long as wide, being wider towards the ventral side (Figs. 2b, 3b, 4b). Both precingular and postcingular series overlap the plates of the apical and antapical series respectively, and inside the series, dorsal plates overlap those more ventrally situated, starting from the dorsal side formed by plates 3'' and 3''' (Fig. 2). The cell division is oblique and one daughter cell keeps plates Po, the four apicals and 1'' and 2'' of the epitheca and 1''', 2''' and 3''' of the hypotheca (Figs. 2,7) After division, the daughter cell that bears the other side which includes the plates that form the ventral right lobe, 5'' and 5''', is very asymmetrical in apical view (Fig. 5c), while the other cell appear more symmetrical with both lobes almost the same size. (Fig.

5d). *G. excentricus* has numerous and small chloroplasts radially dispersed. The nucleus forms an arc in the dorsal side with points towards the ventral side (Fig. 8).

In a different sample from the Canary Islands, *Gambierdiscus* cf. *polynesiensis* was found and was isolated as strain VGO1022. It is smaller in size, has a centrally located Po and a wide 2''''. It will be the subject of a future study.

Ecology and behavior

G. excentricus was found in tidal ponds on rocky shores of volcanic origin in areas very exposed to the intense trade winds of Tenerife, La Palma and Gran Canaria Islands (Fig 1). The cells were on small macroalgae and turf although they were found also in drifting small seaweeds in a protected rocky inlet in La Gomera Island leeward of trade winds. Sea surface temperature in the area ranges from about 18 ºC to 24 ºC and salinity ranges from 36.6 to 36.8 during winter and some years can reach 37 in summer (Neuer et al., 2007). Nevertheless, sampling cannot be considered as representative as it was done opportunistically. In comparison to other *Gambierdiscus* species in culture, *G. excentricus* cultured in our laboratory is a very sedentary species as it is almost non motile, and usually the only appreciable movements are the beaten of longitudinal flagellum and the undulating movement of the transverse flagellum. *G. excentricus* cells were not observed swimming and the two daughter cells usually appear close one to one another after division. In our culture conditions cells appeared more concentrated in the more illuminated areas of the flasks.

Genetics

The phylogenetic results for D1-D3 and D8-D10 LSU sequences are shown in Figs. 9 and 10. Both trees displayed a similar topology confirming that *G. excentricus* sequences clustered into a well supported group, separated from the rest of *Gambierdiscus* species and with *G. australes* as its closest relative. To inspect the differences between *G. excentricus* and the other studied species we calculated the uncorrected genetic distance (p) between the consensus sequences of each species/clade included in the phylogenetic analyses. The minimum number of substitutions per site was obtained for the pair *G. caribaeus*/*G. carpenteri* (0.067 and 0.006 in D1-D3 and D8-D10 original alignments) and *G. yasumotoi*/*G. ruetzleri* (0.009 and 0.008). *G. excentricus* had significantly larger p values (0.350 and 0.083) relative to *G. australes*, its sister group in the analyses. The distance between *G. excentricus* and *G. australes* is also larger than that calculated between *G. toxicus* vs *G. belizeanus* (0.181-0.242 and 0.054 in D1-D3 and D8-D10 original alignments). The D8-D10 sequence of strain VGO1022 was placed in a separate clade which included two sequences from *Gambierdiscus* «ribotype I», as defined by Litaker et

al. (2010). However, the similarity observed between strain VGO1022 and other G. *polynesiensis* sequences in the D1-D3 phylogeny (Fig. 9) indicates that *Gambierdiscus* ribotype I probably belongs to *G. polynesiensis*. Additional work should be carried out to confirm its actual taxonomical status.

Pigment composition

The HPLC chromatogram (Fig. 11) shows the standard peridinin (Per)-containing chloroplast with chl c_2 and Per as major accessory pigments. Chl c_1 was a minor pigment (chl c_1/chl c_2= 0.13) previously detected in the genus *Gambierdiscus* (Durand and Berkaloff, 1985). Diadinoxanthin (Diadino) and dinoxanthin (Dino) are also relevant pigments with different contribution to the carotenoid pool. Pigment ratios respect to chl *a* for carotenoids vary from Per/chl *a* = 1.56, Diadino/chl*a* = 0.41 to Dino/chl *a* = 0.14 while chl c_2/chl *a* = 0.46.

Toxicity

All the strains of *G. excentricus* were toxic to Neuro-2a cells with and without O/V treatment (Table 1). Toxic effects were significantly higher in the presence of O/V treatment ($p < 0.05$) thus indicating the production of CTX-like compounds by the three strains of *G. excentricus* studied.

Table 1
CTX- and MTX-like toxicity estimated using the Neuroblastoma cell-based assay.

CTX-like toxicity

Strain	$IC_{50}^{O/V-}$ ±SD (cells eq. mL^{-1})	$IC_{50}^{O/V+}$ ±SD (cells eq. mL^{-1})	p value (*t* test)	pg CTX 1B eq $cell^{-1}$ ±SD
VGO790	2.11 ± 0.16	0.87 ± 0.10	0.001	1.10 ± 0.19
VGO791	1.60 ± 0.28	0.65 ± 0.23	0.01	1.05 ± 0.18
VGO792	4.58 ± 0.86	2.35 ± 0.77	0.001	0.37 ± 0.17

MTX-like toxicity

Strain	$IC_{50}^{SK\&F\ 96365-}$ ±SD (cells eq. mL^{-1})	$IC_{50}^{SK\&F\ 96365+}$ ±SD (cells eq. mL^{-1})	p value (*t* test)	Dose-ratio (DR)	ng MTX eq $cell^{-1}$ ±SD
VGO790	7.73 ± 0.64	28.81 ± 5.97	0.001	3.73	1.38 ± 0.31
VGO791	14.4 ± 0.33	68.99 ± 24.88	0.02	4.79	0.60 ± 0.24
VGO792	19.78 ± 3.62	71.51 ± 19.43	0.01	3.62	0.48 ± 0.16

All the strains of *G. excentricus* were also toxic to Neuro-2a cells with and without SK&F 96365 treatment (Table 2), with toxic effects significantly different between both treatments ($p < 0.05$). DRs calculated for *G. excentricus* strains were above 1, suggesting the production of MTX-like compounds by the three strains studied.

Estimations of the equivalents of CTX1B and MTX per cells produced by the three strains of *G. excentricus* are given in Table 1. Strains VGO790 and VGO791 produce significantly higher contents of CTX1B equivalent per cells respect to VGO792 (ANOVA, $p<0.01$). Production of MTX equivalents per cells by strain VGO790 was significantly higher than strains VGO791 and VGO792 (ANOVA, $p<0.001$). Additional work with higher amounts of *G. excentricus* biomass obtained from larger scale cultures will be necessary to improve extraction and separation of MTX from CTX to confirm the amounts of toxins produced.

DISCUSSION

Morphology

As already noticed previously by Litaker et al. (2009), a discrepancy exists among different authors on the nomenclatures to describe the tabulation of *Gambierdiscus*. When (Kofoid, 1909) proposed his famous nomenclature system for the plates of dinoflagellates, he chose the names of apical, precingular, postcingular and antapical and intercalary plates in order to have a common criterion of nomenclature. When comparing different genera or species, it is possible to find that homologous plates in different species need to be called with different names if the Kofoid criterion is strictly used. This obviously does not help comparisons. This problem was discussed in the Penrose Conference on «Modern and Living dinoflagellates» held in Colorado Springs, USA in 1978 and several publications followed (Balech, 1980; Eaton, 1980; Edwards, 1990; Taylor, 1979b), which include proposals of new nomenclatural systems that should facilitate the study of homologous plates. A detailed discussion is in (Fensome et al., 1993). Although the new systems facilitate these studies, the modified Kofoid system is still in use and none of the new systems succeeded among conservative neontologists. One of the problems of the Kofoid system concerns the first apical plate (1'), called «1s» or «1u» in the Taylor-Evitt system (Evitt, 1985; Fensome et al., 1993; Taylor, 1979b, 1980) which in some *Alexandrium* species should be called first precingular plate instead of first apical, because it doesn't touch Po. In this case, the formula of the epitheca should be different for different species of the same genus. Due to the toxic character of many of the species of *Alexandrium*, many papers on this genus have been published, and in them it became normally accepted that the first apical plate can be disconnected from Po and is still being called «apical» by most of the authors. In this paper we applied for *Gambierdiscus* a modified Kofoidian

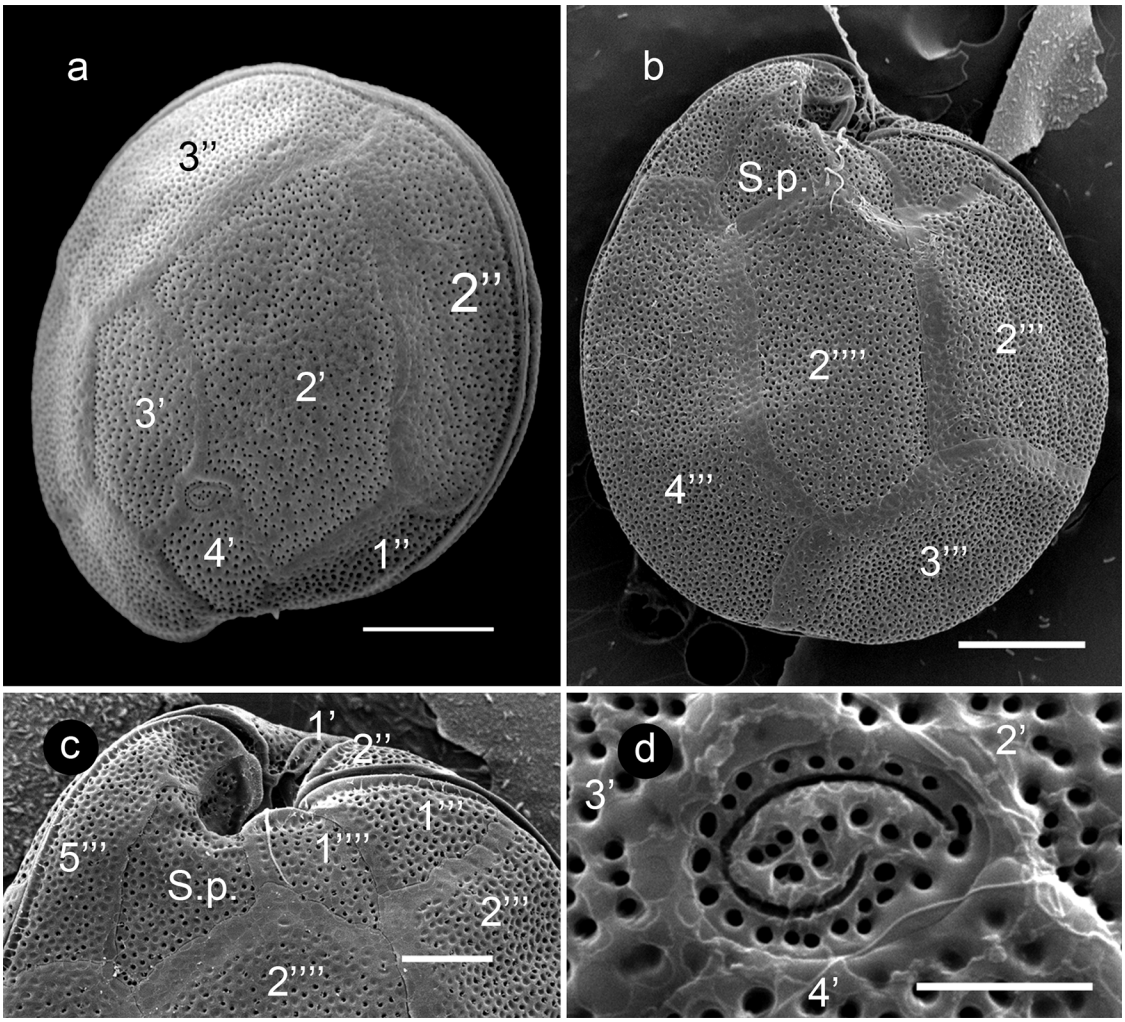

Fig. 4. SEM images of *Gambierdiscus excentricus*. (a) Apical view. (b) Antapical view. (c) Sulcal area. (d) Po plate. Scale bars: (a, b) 20 µm, (c) 10 µm, (d) 5 µm.

nomenclature system as used for *Alexandrium* by Balech (1995) for this genus and by Besada et al., (1982) for *Gambierdiscus, Ostreopsis* and *Coolia*. Gonyaulacales can be grouped in three types according to the plates that contact the homologous to 1' plate (Fensome et al., 1993). If 1' contacts Po, the type is 'insert', if this contact is interrupted by 2' and 4', it is 'metasert' and in the case that the contact between 1' and 2' is interrupted by 1'' it is 'exsert'. In genus *Alexandrium* the three different types can be found: *A. tamarense* is insert, *A. monilatum* is metasert and *A. margalefi* is exsert, so there is no reason to give these plate different names. Plate 1' in *G. excentricus* is minute and does not contact Po being of the exert group of species of Gonyaulacales as *A. margalefi.*

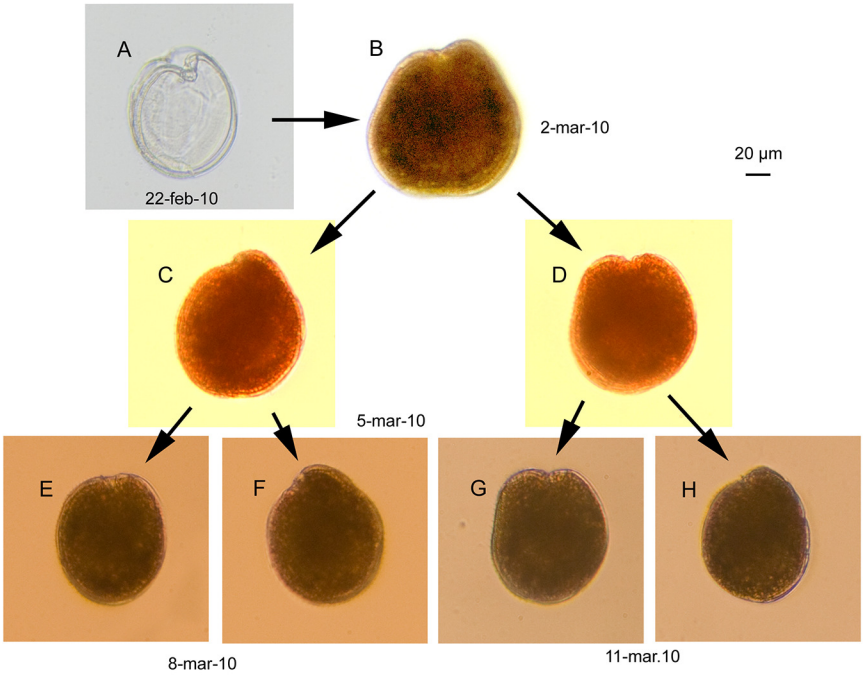

Fig. 5. Morphological differences on descendants of a single cell of *Gambierdiscus excentricus* observed in antapical view. (a) Empty theca after ecdysis. (b) Same cell after 8 days growing. (c and d) Daughter cells after division three days later. (e–h) Third generation of cells. Scale bar. 20 µm.

Similar arguments can be applied to sulcal posterior plate (S.p.) of *Alexandrium,* named «Z» in the Taylor-Evitt system. Its homologous plate is out of the sulcus in *Gambierdiscus*, *Coolia* and *Ostreopsis* (Besada et al., 1982; Taylor, 1979a) as in *Goniodoma sphaericum* (Balech, 1980). On doing this, the plate formula for these Gonyaulacacean genera is the same as follows: Po, 4', 0a, 6'', 6c, ?s, 5'', 0p, 2'', and allows comparisons among them. Plate 1'''' has a wing in the side contacting the sulcus as it happens in *Alexandrium* and *Coolia*, but as in *Gambierdiscus* the ventral area is clockwise twisted, this wing, instead of being faced towards the right side of the cell, is facing the ventral or anterior side. For the same reason, S.p. is displaced to the right side of the hypotheca instead of being central as in *Coolia* and most *Alexandrium* species. Plate 2'''' contacts five plates, 1'''', 2''', 3''', 4''' and S.p. and, like genus *Goniodoma* and *Alexandrium*, and unlike *Coolia* and *Ostreopsis*, it does not contact 5''' (Fensome et al., 1993).

The main character used to differentiate *G. excentricus* from other species of lenticular *Gambierdiscus* is the high ratio between the 2'/3' and 2'/4' suture lengths. Such a morphological character is unique among all the discoid known species of genus *Gambierdiscus* . While in *G. excentricus* this ratio is around 2.3, in the other discoid

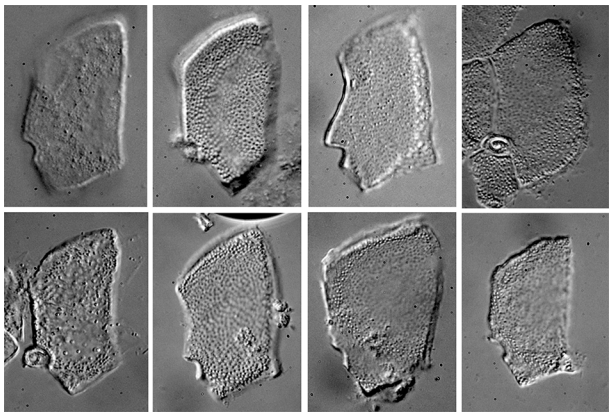

Fig. 6. LM figures of eight Plates 2' of *Gambierdiscus excentricus*.

species ranges between 1.0 and 1.6 . The shape of plate 2' is one of the characteristics used to differentiate species of *Gambierdiscus* (Litaker et al., 2009) and in all the described species the position of Po is more or less centered in the right side of 2', while *G. excentricus* is the only one among the discoid species having it ventrally displaced as in the globular species. This displacement makes that the contact of Po with 2' is also ventrally displaced and then, plate 2' has a peculiar shape (Fig. 6). In a SEM picture of a *Gambierdiscus* cell from the Moroccan coast, not far from the Canary Islands, this characteristic shape of plate 2' was also observed (B. Ennaffah pers. com.). Far from the NE Atlantic, this character was observed in figure 8 of Loeblich III and Indelicato (Loeblich III and Indelicato, 1986) but not in the other figures of the single clone studied by those authors in which this ratio is about 1.5, as the other discoid *Gambierdiscus* species. To explain these morphological differences among cells of the single clone these authors used (F-8), there are two possibilities: one is that their figure 8 shows an aberrant cell as many of the cells shown in other figures, and the other is that more than a clone could exist in that strain corresponding to different species, and one of these being *G. excentricus*. In a sample from Brazil a cell showing this characteristic 2' plate was also observed (S. Nascimento pers. com.), which possibly could be *G. excentricus.* A high parallelism in the Litaker et al., 2009; Richlen et al., 2008) from which it is easily distinguished in base of the shape of 2'.

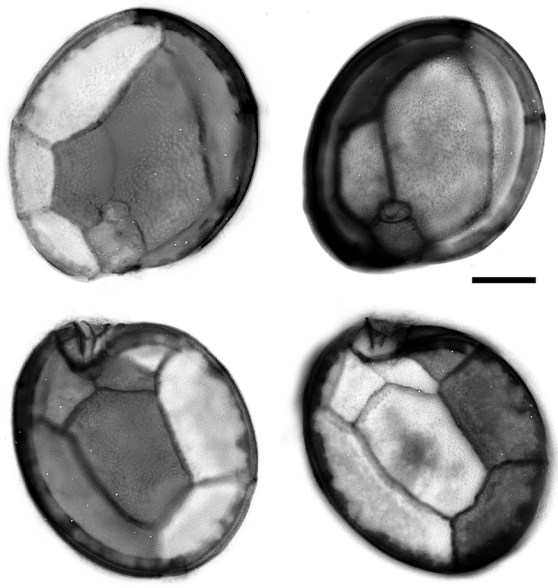

Fig. 7. Calcofluor stained epithecas and hypothecas of cells of *Gambierdiscus excentricus* recently divided in which the different intensity of staining permits the identification of fission lines. Scale bar. 20 µm.

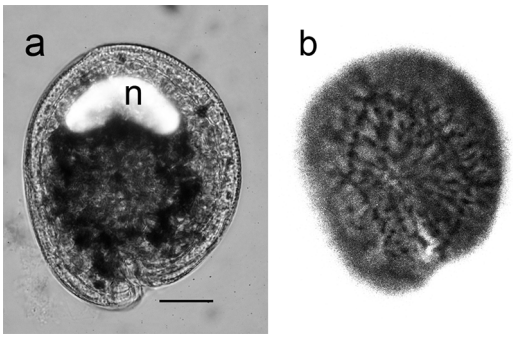

Fig. 8. *Gambierdiscus excentricus*. (a) Epifluorescence image of the U-shaped nucleus in apical view stained with SybrGreen. (b) Epifluorescence image of chloroplasts. Scale bar: 20 µm.

Based on morphology, it can be discounted that the *Gambierdiscus* reported as *Goniodoma* by Silva (1956) in a sample from Cabo Verde, south of the Canary Islands, is *G. excentricus*. Nevertheless it cannot be discounted that Silva's description of Goniodoma could be the same species as strain VGO1022 and close to *G. polynesiensis*.

Phylogeny

LSU generated a robust phylogeny delineating *G. excentricus* as a different specific clade. Both LSU trees were elaborated using selected sequences from two recent comprehensive studies on the genus *Gambierdiscus* (Litaker et al., 2009; Litaker et al., 2010). These authors noted that SSU phylogeny was more informative than LSU for discriminating species at deeper branches, although the resulting topologies were very similar. However, the LSU separation of *G. excentricus* from other related species (e.g. *G. australes*) is solid enough to discount further genetic verification. In a recent study, Litaker et al. 2007 screened the ITS/5.8S variation in 14 genera of dinoflagellates and proposed that uncorrected genetic distance (p) values exceeding 0.04 would represent the boundary at species-level. Based on this approach, Litaker et al. (2009) observed that very closely related *Gambierdiscus* species, as *G. yasumotoi*/*G. ruetzleri*, also fulfilled this rule. Given the higher genetic distance calculated on the basis of LSU phylogenies between *G. excentricus*/*G. australes* in comparison with *G. yasumotoi*/*G. ruetzleri* (see results), it would be also expected that *G. excentricus* displayed p values > 0.04 relative to *G. australes* in a ITS/5.8S alignment. Finally, in certain cases such as for the VGO791 strain, aberrant D1/D3 amplicons were obtained probably corresponding to pseudogene copies of the LSU, as previously noticed in *Gambierdiscus* and other dinoflagellates (Richlen and Barber, 2005; Litaker et al 2009). The D8-D10 sequence from strain VGO1022 matched the two *Gambierdiscus* ribotype I sequences selected in this study (Litaker et al., 2010), not retrieved from cultures until date. These authors suggested that *Gambierdiscus* ribotype I probably represented a new species based on the genetic distances found in D8-D10 region. However, the similarity observed between strain VGO1022 and other *G. polynesiensis* sequences in the D1-D3 phylogeny (Fig. 9) indicates that additional work should be carried out to confirm its actual taxonomical status.

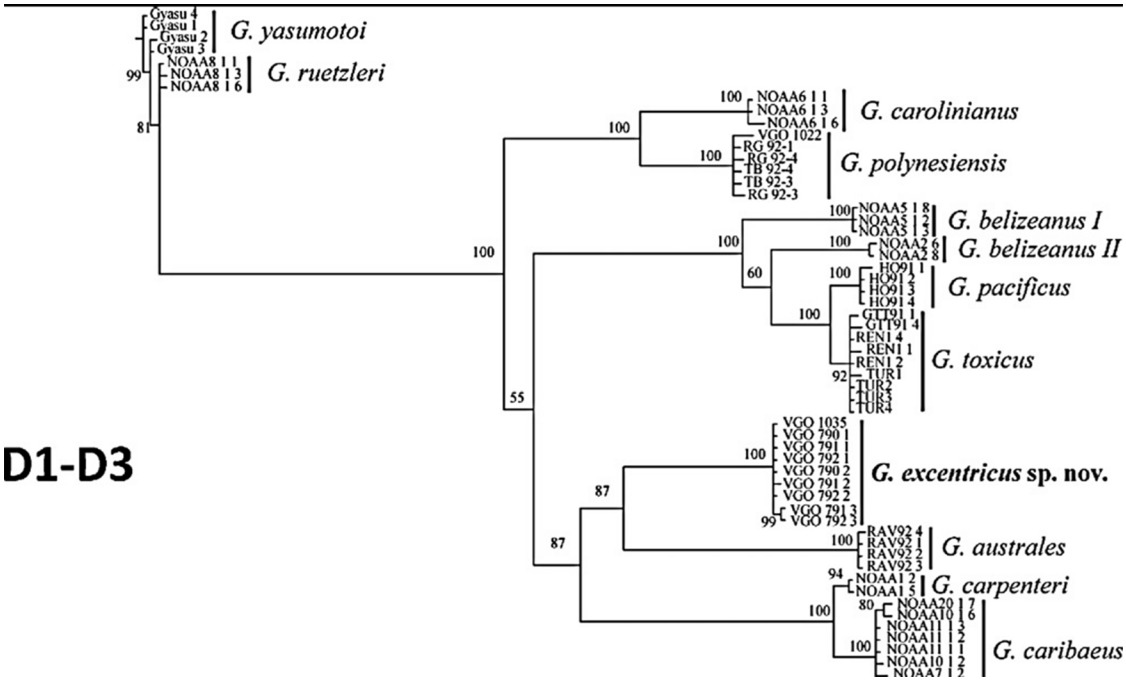

Fig. 9. LSU phylogeny (D1–D3 region) showing the relationship between *Gambierdiscus excentricus* and other *Gambierdiscus* species. The additional numbers that follow each isolate obtained in this study refer to different LSU copies that were PCR amplified and sequenced among descendants from single cells of that isolate. Supports at internal nodes are posterior probability values (Bayesian analyses) and bootstrap values obtained by Neighbor Joining and Maximum Parsimony methods. Hyphens indicate bootstrap values <60. The GenBank accession numbers for the isolates obtained in this study are as follows: *G. excentricus* VGO 790, (GenBank ID: HQ877874) and (GenBank ID: JF303065); VGO 791, (GenBank ID: JF303066-68); VGO 792, GenBank ID: JF303069-71); VGO 1035, (GenBank ID: JF303063), *G.* cf. *polynesiensis* VGO 1022, (GenBank ID: JF303064). Accession numbers from other *Gambierdiscus* sequences are detailed in (Litaker et al., 2009).

Pigments

Peridinin-containing dinoflagellates contain chl c_2 and usually lack chl c_1 (Jeffrey et al., 1975). Only a few dinoflagellate species are exceptions for such a general statement. Chlorophyll c_1 was first detected in *Gambierdiscus* by Durand and Berkaloff (1985) when the separation of chl c_1 and chl c_2 was a methodological challenge. A further study of the pigment composition of *Gambierdiscus* by Indelicato and Watson (1986) described the detailed carotenoid composition; however, they failed to detect chl c_1. The identification of chl c_1 was verified by Bomber et al (1990) using proton nuclear magnetic resonance spectrometry (H^1-NMR). At present the HPLC methods are more selective toward chl *c* separation. All the *Gambierdiscus* strains here studied contained the same pigment pattern with slight difference in quantitative proportions expressed as pigment to chl *a* ratios.

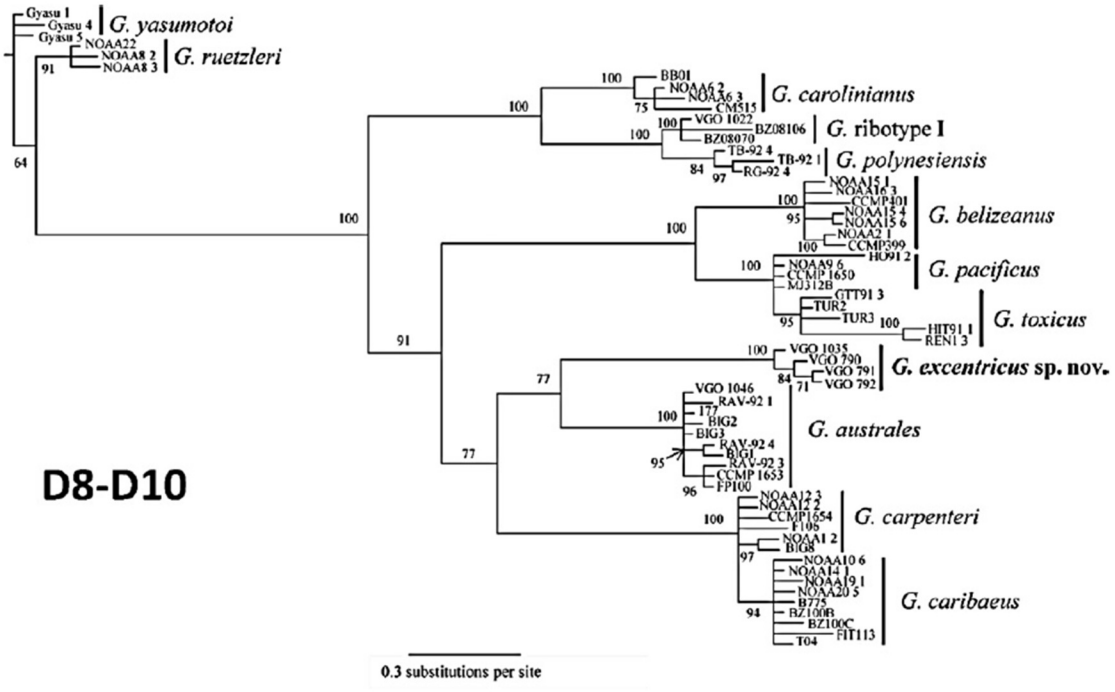

Fig. 10. LSU phylogeny (D8–D10 region) showing the relationship between *Gambierdiscus excentricus* and other *Gambierdiscus* species. The additional numbers that follow each isolate obtained in this study refer to different LSU copies that were PCR amplified and sequenced from single cells of that isolate. Supports at internal nodes are posterior probability values (Bayesian analyses) and bootstrap values obtained by Neighbor Joining and Maximum Parsimony methods. Hyphens indicate bootstrap values <60. The GenBank accession numbers for the isolates obtained in this study are as follows: *G. excentricus* VGO 790 (GenBank ID: JF303074); VGO 791, (GenBank ID: JF303075); VGO792, (GenBank ID: JF303076); VGO 1035, (GenBank ID: JF303073), *G.* cf. *polynesiensis* (labeled VGO 1022), (GenBank ID: JF303077), *G. australes* (VGO 1046, JF303072). Accession numbers from other Gambierdiscus sequences are detailed in (Litaker et al., 2010).

Although the occurrence of chl c_1 was suppressive in peridinin –containing dinoflagellates the simultaneous occurrence of both pigment is not a pigment signature due to other dinoflagellates share this trait.

Toxicity

G. excentricus was identified as a CTX and MTX producer according to the results of the Neuro-2a CBA. The content of CTX1B equivalent per cells quantified for strains VGO790, VGO791 and VGO792 (Table 1) was of the same order as previously reported for other species of *Gambierdiscus* spp. (Caillaud et al., 2010c; Rhodes et al., 2010). As

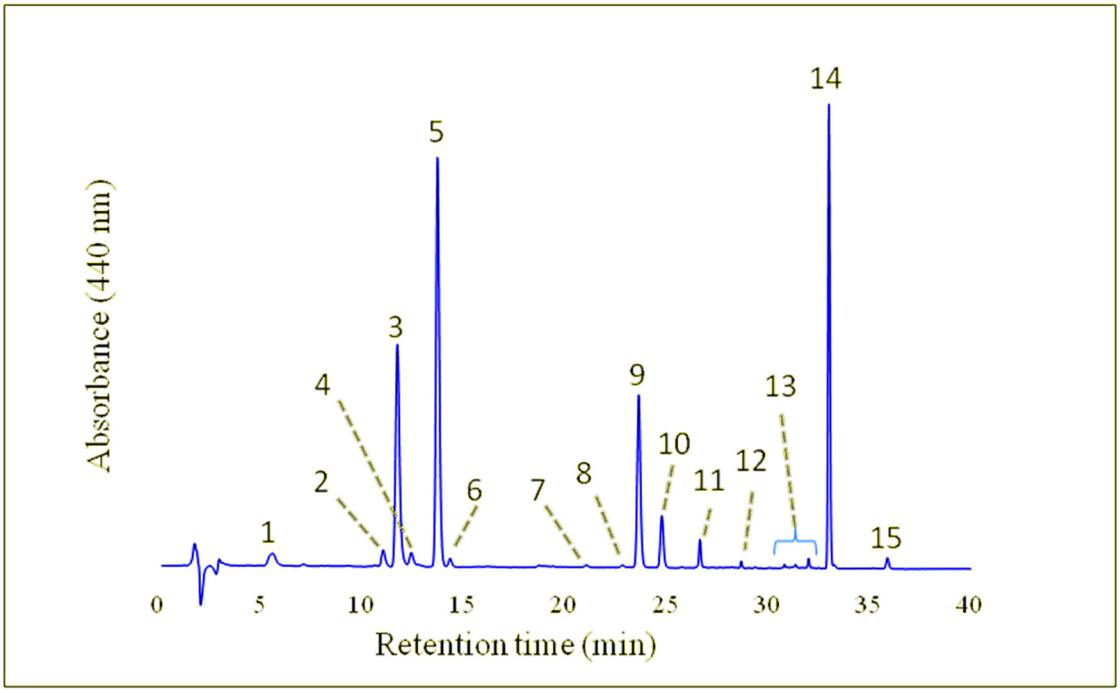

Fig. 11. HPLC chromatogram of *Gambierdiscus excentricus* strain VGO1035. Peak identification: (1) peridininol, (2) divinyl protochlorophyllide (MgDVP), (3) chl c2, (4) chl c1 (5) peridinin, (6) peridinin-like, (7) pyrrhoxanthin, (8) diadinochrome, (9) diadinoxanthin, (10) dinoxanthin, (11) diatoxanthin, (12) unknown carotenoid, (13) chl a allomers, (14) chl a, (15) b,b-carotene. Detection by absorbance at 440 nm.

an example, (Chinain et al., 2010) reported toxicity values according to Receptor Binding Assay (RBA) for *G. toxicus*, *G. australes*, *G. pacificus*, *G. belizeanus* and *G. polynesiensis* from French Polynesia ranging from 0.017 to 11.9 pg CTX3eq cell^{-1} (equivalent to 0.0017 and 1.19 pg CTX1B eq cell^{-1}), *G. polynesiensis* being described as a potent CTXs producer. *G. excentricus* strain production from the Canary Islands ranges between 0.37 and 1.1 pg CTX1B eq cell^{-1}. Regarding the production of MTX, poor data are available in the bibliography regarding the content of MTX produced by *Gambierdiscus* spp. Caillaud et al. (2010c) reported the production of 36.7 nmoles MTX'»10^{-6} cells of *Gambierdiscus* sp from Indonesia, which is equivalent to 0.11 ±0.04 ng MTX cell^{-1}, *G. excentricus* strains from the Canary Islands produce between 0.48 and 1.38 ng MTX cell^{-1} suggesting *G. excentricus* strain VGO790 as a potent MTX producer in relation to the Indonesian strains. However this observation would require the comparison of the MTX production by *G. excentricus* with a higher number of strains/species of *Gambierdiscus* spp. As previously described in the introduction of the present study, the production of MTX by *Gambierdiscus*

spp may not threaten human health (Lewis, 2006). However presence of CTX-producing *Gambierdiscus* spp in a given ecosystem supposes a risk of CFP.

The first ever reported case of CFP in the Canary Islands, Spain (Fig. 1) was caused after consumption of local amberjack (*Seriola rivoliana*) in 2004 (Pérez-Arellano et al., 2005). The *in vitro* Neuroblastoma (Neuro-2a) cell-based assay (CBA) identified CTX-like toxicity and liquid chromatography with mass spectrometry detection (LC-MS) confirmed the presence of Caribbean type 1 CTX (C-CTX-1) together with two other unidentified toxins (Pérez-Arellano et al., 2005). The same year of the intoxications and in an independent study, *Gambierdiscus* sp. was found in the Canarian coast (Aligizaki et al., 2008). More cases were reported in the Canary Islands in 2008 and 2009 caused in both cases by amberjacks that were captured near Selvagem Islands, Portugal (Fig. 1), at 175 km north of Canary Islands, and as in the 2004 case, C-CTX1 was detected by LC-MS/MS (Boada et al., 2010). In these islands several cases of ciguatera were reported but no analyses were done on the meals of the affected people (Gouveia et al., 2009), but several ciguatoxins in addition to C-CTX1 were detected later by LC-MS/MS in amberjacks captured in the area (Otero et al., 2010). Although the presence of *Gambierdiscus* and the cases of ciguatera in the East Atlantic were only recently reported, this was probably due to lack of studies. *Gambierdiscus* sp. was observed in the area as early as 1948 in the Cabo Verde archipelago, although reported as *Goniodoma* sp. (Silva, 1956) and it can be considered as the first record of this genus. Comparing the figure of Silva (1956) with *G. excentricus* we can conclude that they correspond to different species of *Gambierdiscus* but, nevertheless the species of Cabo Verde could be the same as the second species found in Canaries but more studies are necessary. The first historic record of ciguatera in the world could be also from the Eastern Atlantic. In 1525, at the beginning of the second circumnavigation of the world, a fleet of seven Spanish ships anchored in the island of San Mateo, which probably corresponds to which today is known as Annobon, in the Gulf of Guinea. The direct translation from the original report in Spanish says: «On this island, a very beautiful fish was caught in the flagship, called barracuda, and the Captain General invited some of the captains and officers of the King. All who ate the barracuda fell ill from diarrhea and were unconscious, so we thought they had died; however our Creator wanted everyone to be saved.» (Urdaneta, 1580). This incident was considered very important in its time as it was described with similar words in other reports of the same travel. As all the captains who were poisoned, died during this cruise few months later of unknown causes different from the common and well known scurvy, ciguatera is considered as a probable cause of their dead (de Miguel, 2009). Among the dead, was Juan Sebastián Elcano who was the first captain to circumnavigate the world only few years before. The recent identification of ciguatoxins in fishes of Cameroon (Bienfang et al., 2008), very near of the Island of Annobon, and the fact that the

intoxications were caused by a big barracuda, support the consideration of these poisonings to be the first record of an outbreak of ciguatera in the world.

ACKNOWLEDGEMENTS

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GENUS *GAMBIERDISCUS* IN THE CANARY ISLANDS (NE ATLANTIC OCEAN) WITH DESCRIPTION OF *GAMBIERDISCUS SILVAE* SP. NOV., A NEW POTENTIALLY TOXIC EPIPHYTIC BENTHIC DINOFLAGELLATE.

ABSTRACT

Species of the dinoflagellate genus *Gambierdiscus* are the cause of Ciguatera Fish Poisoning which is very common in some tropical areas. Nevertheless, until recently this disease was not reported in the NE Atlantic Ocean. A new photosynthetic dinoflagellate species, *Gambierdiscus silvae* sp. nov. is described based on samples taken from tidal ponds on rocky shores of the Canary Islands (NE Atlantic Ocean). Its morphology was studied by light and scanning electron microscopy. It is anterioposteriorly compressed, lenticular in shape with an epitheca higher than hypotheca. It is round in apical view and has a thick smooth theca with many scattered pores. Plate 2' is hatchet shaped and plate 2'''' is very wide and the biggest of hypotheca. Phylogeny inferred from the large subunit nuclear rRNA (D1-D3 and D8-D10 regions) showed that *G. silvae* strains (VGO1022 and VGO1167) clustered in a well supported sister clade to *G. polynesiensis*, distinct from the other species. *G. australes* was observed in these samples (the first record for this species in the Atlantic), together with *G. excentricus* already reported to these islands. This work increases the number of described species of genus *Gambierdiscus*, and shows an unexpected diversity of this genus in the Canary Islands.

INTRODUCTION

Ciguatera fish poisoning is a syndrome caused by eating some toxic marine fish from tropical and some temperate areas. It is caused by toxins produced by dinoflagellates of genus *Gambierdiscus* Adachi & Fukuyo which are metabolized and transmitted through the food web to humans (GEOHAB, 2012, Parsons *et al.*, 2012). The name ciguatera is from Cuban origin where this syndrome was already well known long time ago when the symptoms were precisely described, and warnings were given about which fish species were edible and which were susceptible of causing ciguatera (Parra, 1787). Ciguatera was considered as an only tropical syndrome, although in temperate countries some intoxications caused by imported fish were reported. But recently, some cases after consumption of local fishes have been reported in temperate areas like the Canary Islands

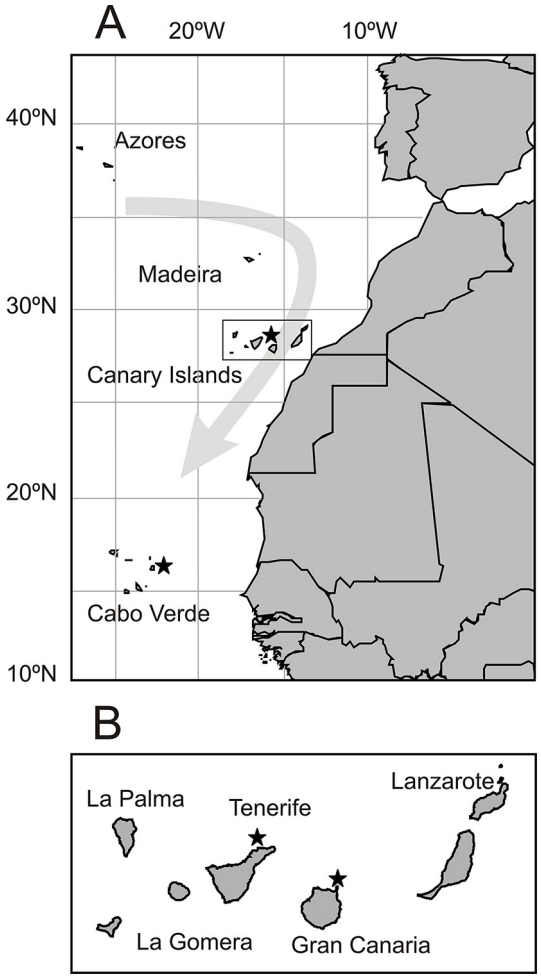

Figure 12. A) Map of the East Atlantic archipelagos. B) Map of the Canary Islands. Stars: Localities where Gambierdiscus silvae was found in Canary Islands and Goniodoma sp. in Cabo Verde

(Pérez-Arellano et al., 2005, Boada et al., 2010). Species of genus *Gambierdiscus* were also reported from non tropical seas only recently (Fraga *et al.*, 2011, Nishimura *et al.*, 2013, Aligizaki & Nikolaidis, 2008, Aligizaki et al., 2008). Ciguatera affects mainly the Caribbean Sea and Polynesia and it is considered the most common seafood poisoning of non bacterial origin, with about 20,000 to 60,000 cases per year in the world (Yasumoto, 2005). Although the mortality rate is very low, the affected people can remain ill for long periods of time and can become a chronic disease (Pearn, 1994). Although the Eastern Atlantic Ocean was not considered an affected area, the oldest historical record of ciguatera was described from the Gulf of Guinea in 1521 (Urdaneta, 1580), and the first world's report of an observation of cells of *Gambierdiscus* (although referred as *Goniodoma* sp.), dated from October 1948 near the coast of Boavista Island, in the Cabo Verde archipelago (Silva, 1956).

Genus *Gambierdiscus* was erected by Adachi & Fukuyo (1979) with *G. toxicus* as the type species. It was considered as a monospecific genus for many years but up to 12 species were described until now (GEOHAB, 2012, Parsons et al., 2012, Nishimura *et al.*, 2014). Based on the information given by all these species, *G. toxicus* has had to be re-described as it was observed that the original description was done with more than one species (Litaker et al., 2009). In fact, it is quite common to observe several species of *Gambierdiscus* in the same area (GEOHAB, 2012, Litaker et al., 2010, Nishimura et al., 2013, Parsons et al., 2012, Xu *et al.*, 2014) and the morphological differences among them are usually very subtle making difficult to recognize their diversity in a particular place.

In this study, new information on the presence of *Gambierdiscus* in the Canary Islands is given with the first report of the *G. australes* in the Atlantic Ocean and the description of a new species, *Gambierdiscus silvae* sp. nov., on the basis of morphology and genetic sequencing of cultured strains isolated in the Canary Islands. This species (formerly known as *G*. sp. ribotype 1), turns out to be morphologically different from the other species of *Gambierdiscus* already described and genetically different from other *Gambierdiscus* species and phylotypes. We suggest that *G. silvae* is probably the species reported by Silva (1956) from Cabo Verde archipelago as *Goniodoma* sp.

RESULTS

Three species of genus *Gambierdiscus*, *G. excentricus* S. Fraga, *G. australes* M. Chinian & M.A. Faust and *G. silvae* sp. nov. were found in the Canary Islands (Fig. 12). *G. excentricus* were already reported in the area as it is the type locality (Fraga et al., 2011), but this is the first report of *G. australes* in the Atlantic Ocean as until now it had been only recorded in the Pacific Ocean (Chinain et al., 1999, Litaker et al., 2010, Nishimura et al., 2013).

The three species were found in tidal ponds in the rocky shore which means that they can share the same ecological niche. *G. excentricus* was observed in few samples obtained in lower level tidal ponds, but *G. silvae* and *G. australes* were observed also in high level tidal ponds.

Morphology

Cells of *G. silvae* are anterioposteriorly compressed, lenticular in shape with the epitheca taller than the hypotheca and symmetrical in ventral view (Figs. 13C, 14C), 46 ± 5 µm long, 69 ± 8 µm deep, 64 ± 9 µm wide. It is round in apical and antapical view (Figs. 13A, B, 14A, B) and has a thick theca with many scattered pores. Apical and antapical plates are smoother than pre- and postcingular plates that show a pattern of shallow depressions more visible near the cingulum (Fig. 13A, B, 14A, B, 15A, 16B). Plate formula is Po, 4', 0a, 6'', 6c, ?s, 5''', 0p, 2''''. Apical pore complex, Po is oval, has a fish hook slit, and it is centred in the epitheca, only slightly ventrally displaced (Fig. 13A, D). Plate 1' has the shape of an arrow head (Fig. 15B), it is very small and is not in contact with Po. Plate 2' is hatchet shaped and it is usually the biggest of the epithecal plates. In old cultures, where couples of fusing cells were observed, there are many tall cells, almost spherical, with pre- and postcingular plates elongated in the apical axis, while apical and antapical plates have the same size as common cells (Fig. 15C-D). In most of the cells, the sutures 2'/1'' and 2'/2'' do not form an angle but a continuous curve (Figs. 15D, E, G) giving plate 2' the shape of a pointed axe. The suture 2'/3'' is about

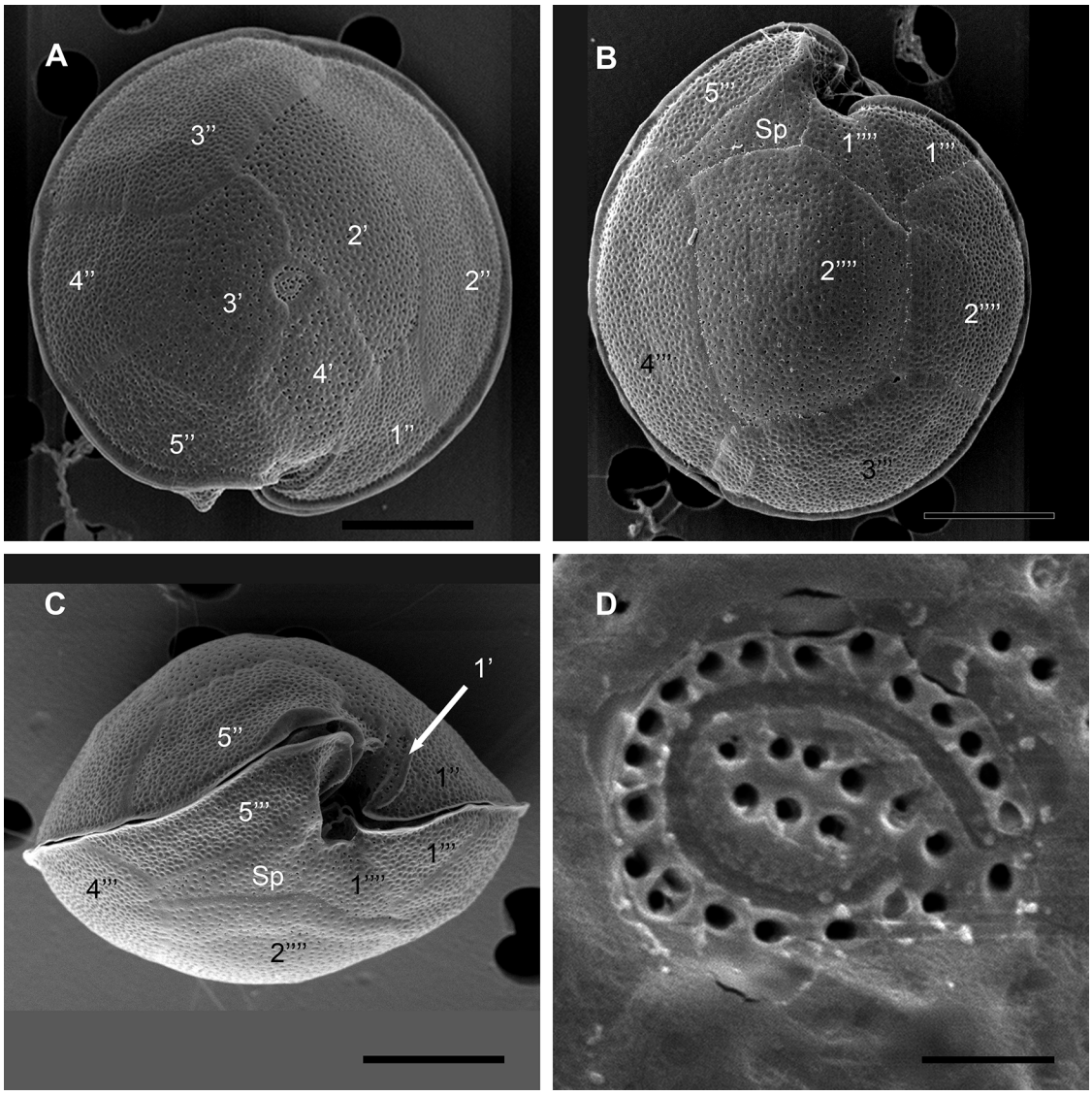

Figure 13. *Gambierdiscus silvae*. SEM images A) Apical view, B) Antapical view, C) Ventral view, D) Po plate. Scale bars: 20 µm, except D, 2 µm.

twice as 2'/1''. Plate 3' is about same size as 4'. Precingular plates have different sizes but 2'' is the wider and can be the biggest of the epitheca in tall cells (Figs. 15A, C, D). Plate 3'' is asymmetrical having suture 3''/2' longer than 3''/3'. Plate 6'' is very small, and with 1', it forms the anterior edge of the sulcus (Figs. 13C, 4B). Plates 1', 4' and 1'' do not form a distinct fold like in *Gambierdiscus polynesiensis*, as described by Litaker et al (2009). Cingulum is narrow and deeply excavated. The sulcus is like a funnel with the

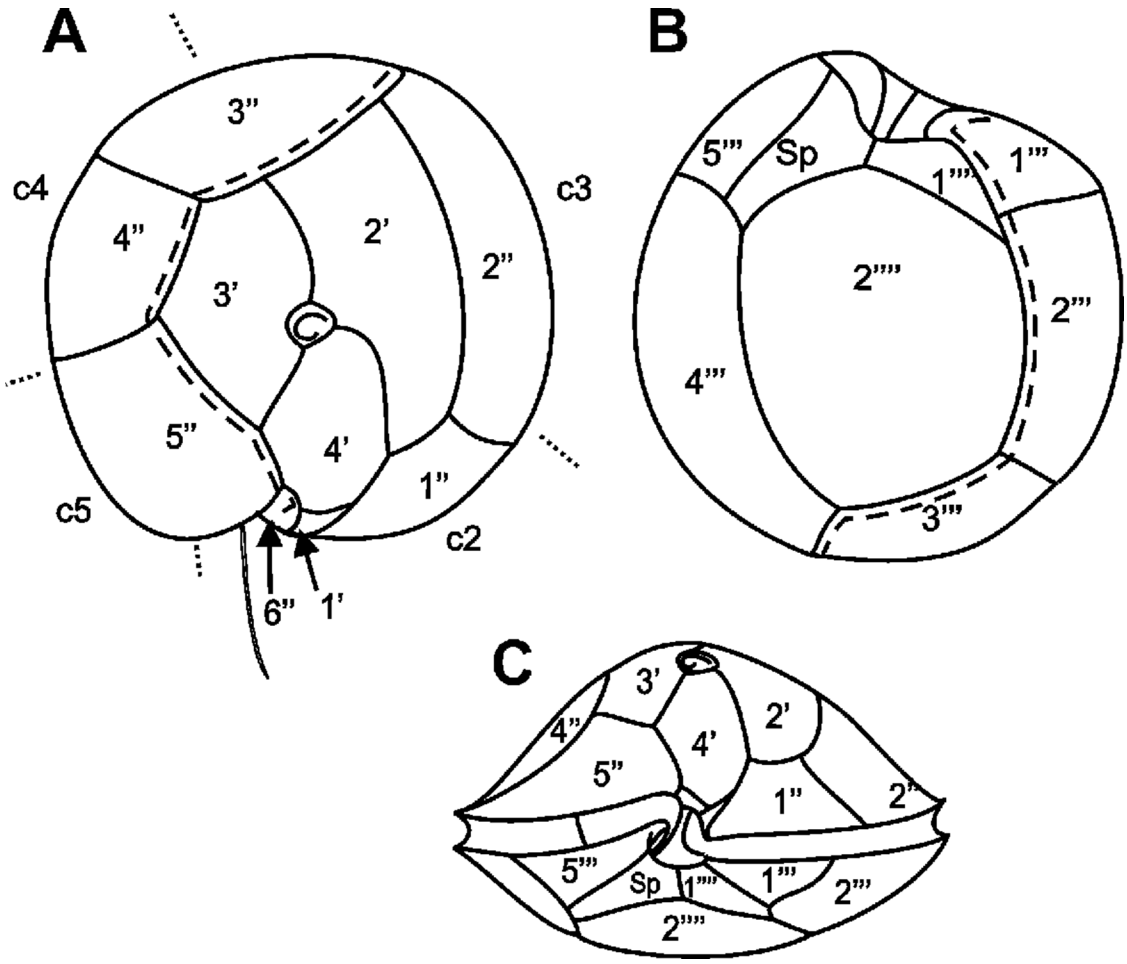

Figure 14. Ink drawings of *Gambierdiscus. silvae*. A) Apical view, B) Antapical view, and A) Ventral view. Dashed lines indicate scission line..

longitudinal flagellum radially orientated in the equatorial plane instead of being posteriorly orientated as in most dinoflagellates. For this reason the sulcus is very short and S.p. plate is outside of the sulcus (Figs. 13B,C, 14B, C, 16A, B).

In the hypotheca, plate 2'''' is very wide (Fig. 13B, 14B, 16A-C) and usually occupies most of the hypotheca in a way that all the other plates appear like compressed to the periphery of the hypotheca (Figs. 16A). Nevertheless, in tall cells the relative size of 2'''' is smaller compared as it is in common cells. Plate 1''' usually does not contact 2'''' but sometimes these two plates plus 1'''' and 2''' meet in one point. In a similar way, 2'''' is usually not in contact to 5''' (Fig. 13B, 16A) but in some cases these two plates have a common suture, and plate 2'''' is six sided instead of five sided (Fig. 16B).

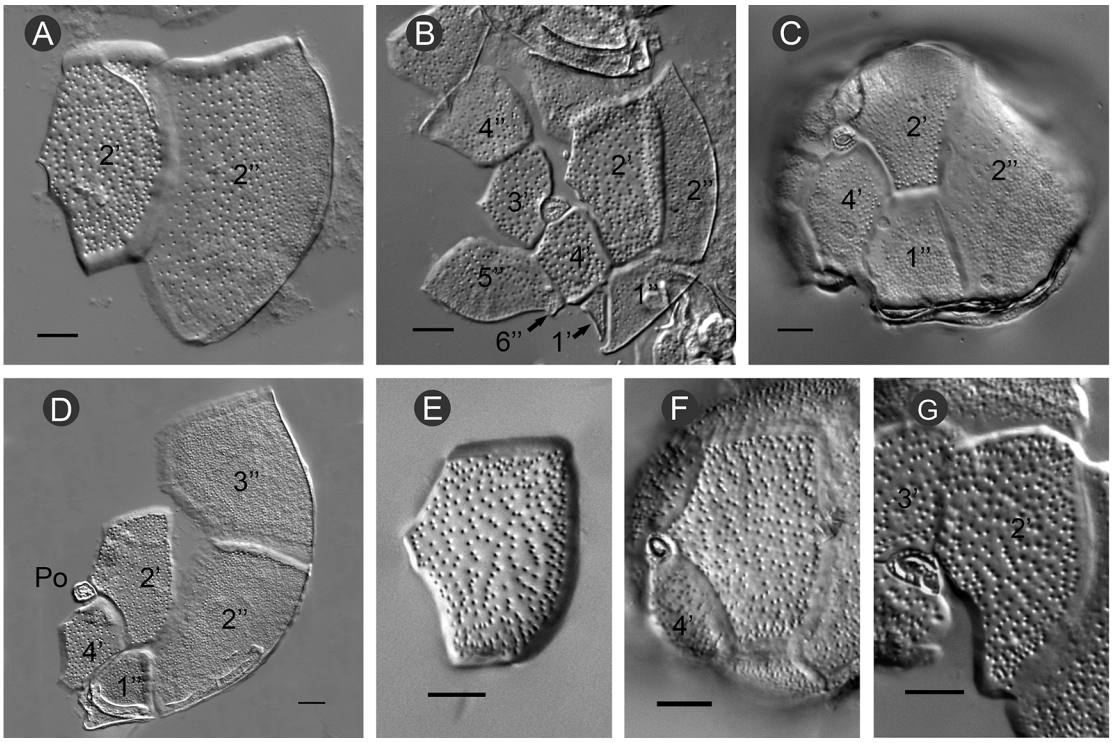

Figure 15. *Gambierdiscus silvae*. LM images of epithecal plates. A) Plates 2' and 2''. B) Disected epithecal plates. C) Left side of the epitheca of a tall cell. D) Squashed epitheca of a tall cell with big precingular plates. E-F) Plate 2'. All scale bars: 10 µm.

Cells of *G. silvae* are photosynthetic and have numerous branched chloroplasts.

The cells of *G. australes* matched the morphological characteristics of the species (Chinain et al., 1999, Litaker et al., 2009) namely, rectangular plate 2', narrow 2'''', asymmetric 3'' and smooth surface (Fig. 17). The size of the *G. australes* cells from Tenerife have an average length 46 ± 6 µm, depth 85±8 µm , and width 76±7 µm.

Distribution and ecology

G. silvae was found associated with *G. excentricus*, *G. australes*, *Prorocentrum lima, Prorocentrum* spp. *Ostreopsis* spp., *Coolia spp*., *Bysmatrum* sp., *Amphidinium* sp. and *Heterocapsa* sp. as epiphytic on a mixture of small seaweeds in rocky tidal pools on the NW coasts of the islands of Gran Canaria and Tenerife, in areas exposed to the strong dominant trade winds. Seawater temperature in the area ranges from 18 to 26 ºC and has a yearly average of about 21 ºC (Borges *et al.*, 2004). The day of sampling in La Puntilla,

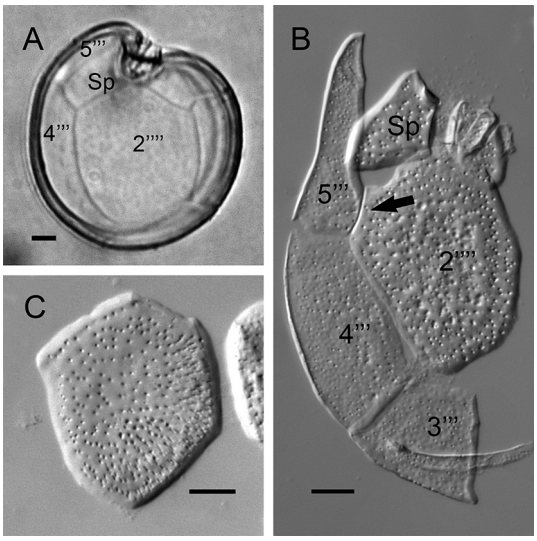

Figure 16. *Gambierdiscus silvae*. LM images of hypothecal plates. A) Whole hypotheca with a wide plate 2'''' which is not in contact to plate 5'''. B) Several dissected hypothecal plates showing 2'''' in contact to 5'''(arrow). C) Plate 2''''. All scale bars: 10µm.

winds over 120 km'»h^{-1} were recorded. During sampling in Punta Hidalgo the weather was sunny and calm and seawater temperature was between 23 and 24ºC.

Phylogenetic analyses

Two phylogenetic trees based on D8-D10 and D1-D3 regions of the LSUrRNA gene were elaborated (Figs. 18-19). Both phylogenies reconstructed the same relationships between clades of *Gambierdiscus* species (and ribotypes/sp. types not formally described as species). Sequences of *G. silvae* (VGO1022 and VGO1167) clustered together as a separate group, sister to *G. polynesiensis*. In the D8-D10 phylogeny (Fig. 18) the alignment could include several sequences from field isolates, labelled as *G.* sp. ribotype 1, which grouped together with *G. silvae* from the Canaries. In the D1-D3 phylogeny (Fig. 19) the *G. silvae* clade was built only with the sequences from Canaries isolates, as no other reports for this species could be retrieved from GenBank.

The genetic distances between groups (net distances, see methods) were calculated in both phylogenies. Their values for the *G. silvae-G.polynesiensis* pair were of 0.012 and 0.047 (using D8-D10 and D1-D3 sequences, respectively), and of 0.015 and 0.196 between

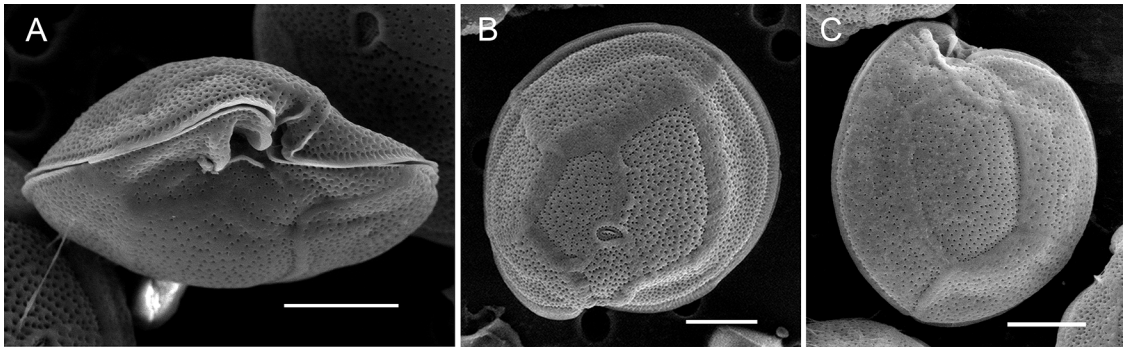

Figure 17. ***Gambierdiscus australes.*** SEM images A) Ventral view, B) Apical view, C) Antapical view. All scale bars: 20µm.

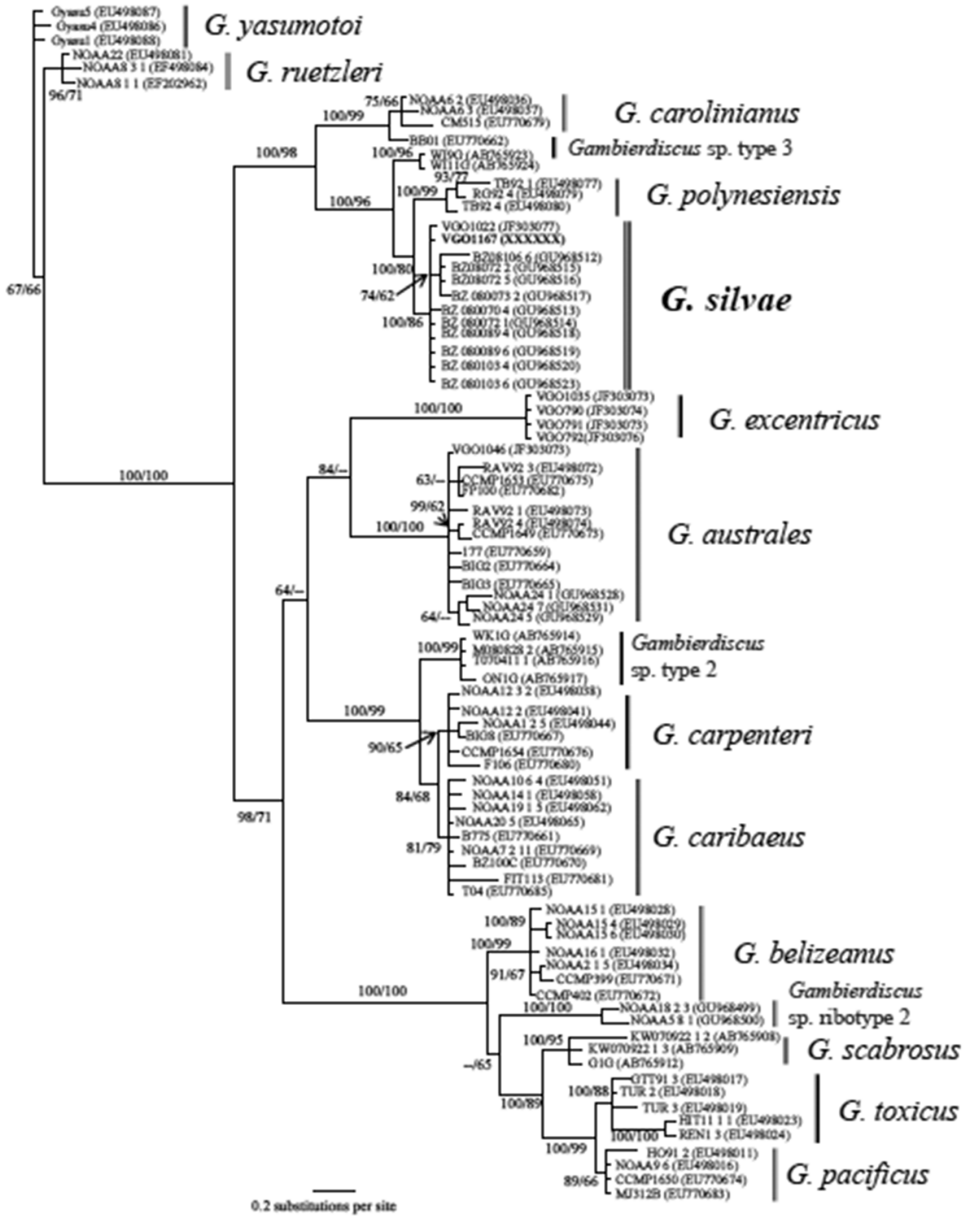

Figure 18. LSUrRNA phylogeny (D8–D10 region) showing the relationship between *Gambierdiscus silvae* and other *Gambierdiscus* species/phylotypes. New sequences from this study are in bold. Strain names and GenBank Acc.Nos. are detailed in each case. Internal nodes supports are posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens indicate bootstrap values <60.

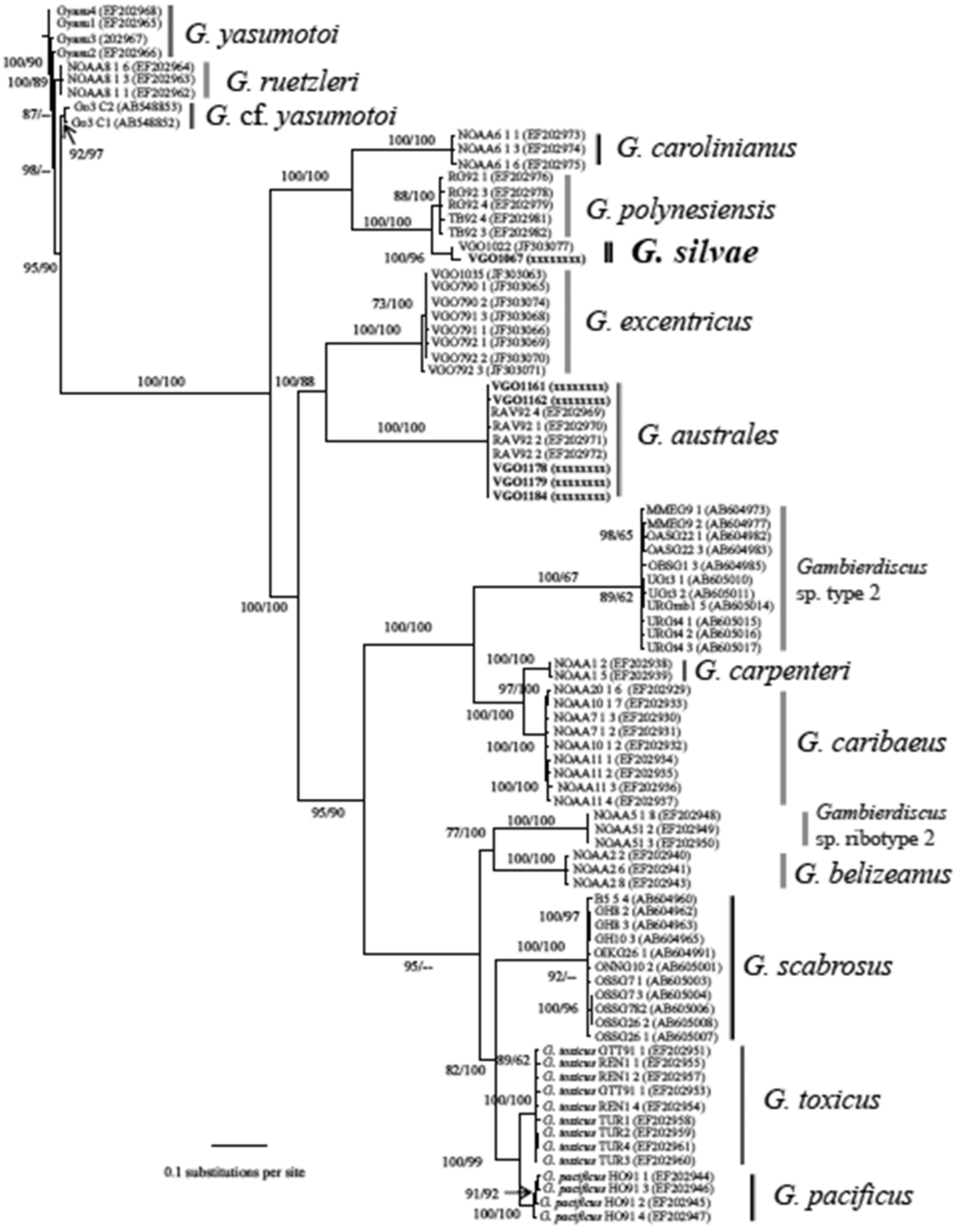

Figure 19. LSUrRNA phylogeny (D1–D3 region) of genus *Gambierdiscus,* including *Gambierdiscus silvae* and *G. australes* from Canary islands and other *Gambierdiscus* species and phylotypes. New sequences from this study are in bold. Strain names and corresponding GenBank Acc.Nos. are detailed in each case. Internal nodes supports are posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens indicate bootstrap values <60.

G. silvae-G. sp. type 3. These genetic distances were similar or larger to those observed between close *Gambierdiscus* species, like *G. yasumotoi-G. ruetzleri* (0.005 and 0.009), *G. carpenteri-G. caribaeus* (0.005 and 0.052) or *G. toxicus-G. pacificus* (0.008 and 0.040).

DISCUSSION

Distribution and ecology

G. excentricus, that was already known from the area which is its type locality (Fraga et al., 2011) was found together with *G. australes* which were not reported before in the Atlantic Ocean (Litaker et al., 2010, Parsons et al., 2012) and with the new species *G. silvae* which we think it is probably the same species reported by Silva (1956) from further south, in Cabo Verde archipelago. Canary Islands are not an exception in having more than two species of *Gambierdiscus* in the same area as this was observed as well in French Polynesia (Chinain et al., 1999), Belize, Puerto Rico (Litaker et al., 2010), Japan (Nishimura et al., 2013) and Kiribati (Xu et al., 2014). Places with two species are very common (Litaker et al., 2010). It is interesting to note that the three species found in the Canary Islands have three different biogeographies. 1) *G. excentricus* was only observed in temperate areas of the Atlantic Ocean, namely the Canary Islands (Fraga et al., 2011), the Moroccan coast (B. Ennaffah pers. comm.), (Parsons et al., 2012) and Brazil (Nascimento *et al.*, 2012) but not in the Caribbean where other five *Gambierdiscus* species and two ribotypes were reported (Litaker et al., 2010). 2) *G. australes* which was reported from the Pacific Ocean, in French Polynesia, Hawaii and Japan and in the Canary Islands, being also absent from the Caribbean Sea. And 3) *G. silvae* which was only observed in the Atlantic Ocean, as in addition to the Canary Islands, it was reported from the Caribbean Sea as Ribotype 1 (Litaker et al., 2009). Xu et al (2014) described *Gambierdiscus* sp. Type 4 from the Pacific Ocean which is both genetically and morphological very close to *G. silvae*. Further studies are necessary to decide if *Gambierdiscus* sp. Type 4 is the same species as *G. silvae* or they represent two species that could have recently diverged in a sympatric process of speciation after the closing of the Isthmus of Panama about 3 Ma as it was observed in genus *Ostreopsis*, another warm water benthic dinoflagellate (Penna et al., 2010).

The coexistence of congeneric species with apparently the same ecological niche is an outstanding topic that has attracted the attention of ecologists (Levine & HilleRisLambers, 2009). The Canary Islands coastal waters have very low seasonal changes in temperature, so, in general, it is a stable environment, but in the tidal ponds the environmental changes are huge and quick. Hutchinson's (1961) plankton paradox can be explained in this highly variable environment, where the time for environmental changes is much smaller than the generation time of the cells, and hence, there is no competition

among cells for the resources. In parallel to genus *Gambierdiscus*, several species of *Coolia* and benthic *Prorocentrum* were also found in the same samples. In this case it looks that there are no competition for resources among congeneric species and that the neutral theory of biodiversity proposed by Hubbell (2001) could be applied.

Morphology

G. silvae has a hatchet shaped plate 2', an asymmetrical 3'' and a broad 2'''', as *G. polynesiensis* and *G. carolinianus*. It is very similar to *G. polynesiensis* in general shape and tabulation but it differs from it in 1) the lack of the distinct fold of the later, formed by 4', 1' and 1'' (named as 1', 1'' and 2'' in Litaker et al. (2009)), 2) on the shape of Po, which is oval in *G. silvae* and triangular in *G. polynesiensis,* 3) on the ventral view which is symmetrical in *G. silvae* and asymmetrical in *G. polynesiensis* and 4) in having different plate textures. *G. silvae* differs from *G. carolinianus* in the shape of 1' which in *G. carolinianus* is shorter than in G. *silvae*. *G. carolinianus* is also wider than deep while *G. silvae* is deeper than wide.

The species reported by Silva (1956) as *Goniodoma* sp. (Fig. 20) was probably *G. silvae*. No differences can be observed in the epitheca or in ventral view. The drawing of the hypotheca is more difficult to interpret as, although it is not optically reversed, it is observed from inside like figures 4B and 4C of Nishimura et al (2014) in a way that cingular and some sulcal plates are overlapped to the hypothecal plates and hence, hiding plates 5''' and 1''''. Plate 2' is hatchet shaped and the sutures with 1'' and 2'' are in a continuous curved line. Plate 2'''' is wide.

The presence of tall cells in cultures where coupling cells were observed suggest that these tall cells could be planozygotes. Morphological differences in plates shapes of

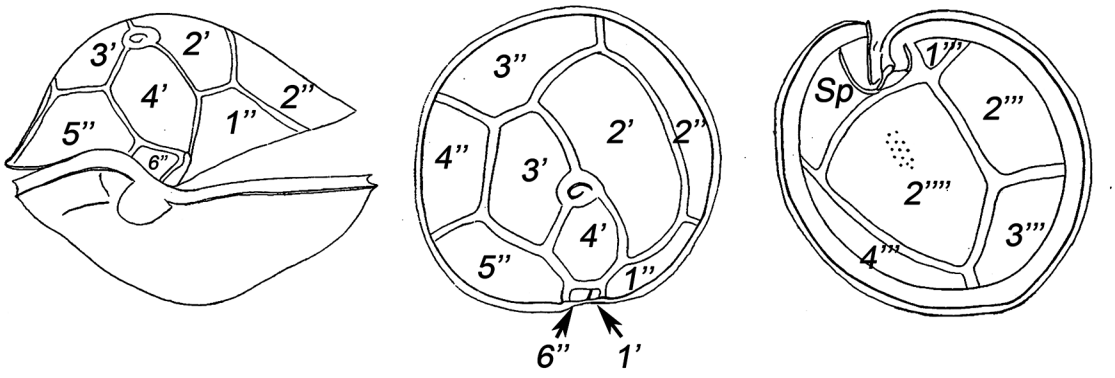

Figure 20. *Goniodoma* sp. modified after Silva (1956) with plates numbers added according to the criteria followed in this paper.

different life cycle stages have been reported in another genus of Gonyaulacales, *Fragilidium*, where in vegetative cells of *F. subglobosum* and *F.* cf. *duplocampaneforme*, plate 1' does not contact Po, while in gametes, these two plates are in contact (Amorim *et al.*, 2013). In the case of *Gambierdiscus* the zygotes could have a bigger volume than vegetative cells incrementing the height of precingular and postcingular plates while keeping the same shape and size of apical and antapical plates.

Gambierdiscus sp. type 4 as described by Xu et al. (2014) looks like *G. silvae* en their Figures. 5A and C by the shape of 2' and the difference in texture between apical and precingular plates, but the hypotheca looks different as plate 2'''' (their 1p) looks not as wide as in *G. silvae*.

The cells of *G. australes* from Tenerife are coincident with the original description from the Pacific Ocean (Chinain et al., 1999) both in morphology and dimensions.

Phylogeny.

The phylogenetic relationships between *Gambierdiscus* species and phylotypes in our study were similar to those reported by other authors, including also our research group (Xu et al., 2014, Nishimura et al., 2014, Fraga et al., 2011, Litaker et al., 2010). In the last years, several new genetic clades have emerged and could represent new undescribed *Gambierdiscus* species, pending of a formal description to confirm their taxonomic status as valid species. Before the present study, eight of these genetic groups (termed as *Gambierdiscus* sp. ribotypes 1 and 2 (Litaker et al., 2009) and six *G*. sp. types (types 1 to 6 following Nishimura et al. (2013) and Xu et al. (2014) were found in the literature. Regarding *G. silvae* (previously *G*. sp. ribotype 1), the first sequences were reported by Litaker (2010) from field isolates in Belize (Caribbean Sea) and there existed a single cultivated strain (VGO1022) as reported by Fraga et al (2011). The genetic relationships for *G. silvae* were already shown by Litaker et al. (2009), Nishimura et al. (2013) and Xu et al. (2014)), but referring instead to *G*. sp. ribotype 1. The new genetic data presented in our study includes a second strain (VGO1167) of *G. silvae* (whose D1-D3 and D8-D10 LSUrRNA sequences resulted almost identical to the first isolate), together with sequences of four isolates of *G. australes*. Recently, Xu et al. (2014) reported a new phylotype (*Gambierdiscus* sp. type 4) in an intensive sampling in Kiribati Islands (Pacific), close to *G. silvae* and these authors indicated that it may represent a Pacific clade of *Gambierdiscus* sp. Ribotype 1. Even if LSUrRNA demonstrates a good resolution between *Gambierdiscus* species and genetic clades until now, as new studies continue adding complexity to the specific diversity in this genus, it would be advisable to explore other molecular markers and expand current LSUrRNA trees to others based on ITS or

mitochondrial genes, as it has been extensively examined to better delineate species in other dinoflagellate genera.

Gambierdiscus silvae *sp. nov. Fraga & Rodríguez.*

Description: Cells anterioposteriorly compressed, lenticular in shape with an epitheca higher than hypotheca with averaged length 46 ± 5 µm, depth 69 ± 8 µm, and width 64 ± 9 µm. It is round in apical view and has a thick smooth theca in apical and antapical plates, and slightly ornamented in pre- and postcingular plates, with many scattered pores. Plate formula is Po, 4', 0a, 6'', 6c, ?s, 5''', 0p, 2''''. Apical pore complex, Po is oval, has a fishhook-shaped slit, is centred in the epitheca and only slightly ventrally displaced. Plate 2' is hatchet shaped and is the biggest of the apical plates. Plate 2'''' is the biggest of hypotheca and is very wide in relation to the length. The nucleus is U shaped and is located in the dorsal part of the cell with points towards the ventral side of the cell. Cells are photosynthetic.

Etymology of the epithet: This species is dedicated to the late Dr. Estela Sousa e Silva, a Portuguese researcher who was the first in the world to report a *Gambierdiscus* species from samples taken in Cabo Verde in 1948 although as *Goniodoma* sp..

Holotype: Figure 13A obtained from clonal strain VGO1022 deposited in the Culture Collection of Harmful Microalgae (CCVIEO) of the Centro Oceanográfico de Vigo of the Instituto Español de Oceanografía, Vigo, Spain. Strain VGO1022 barcoded in GenBank accession numbers JF303077.1 and JF303064.1 Clone VGO1022 was collected on February 18th, 2010 as an epiphyte on small filamentous macroalgae and turf on a tidal pond in La Puntilla, Las Palmas, Canary Islands (Fig. 12).

Type locality: La Puntilla, (28º 8,89' N, 15º 25,95' W) Las Palmas, Canary Islands, NE Atlantic Ocean.

Habitat and distribution: Marine, associated as epiphyte to seaweeds in a rocky tidal pool. It was observed in two of the Canary Islands, Tenerife and Gran Canaria. It is probably the same species that Silva (Silva, 1956) reported from near Boavista Island, Cabo Verde, another Macaronesian archipelago. Based on the D8–D10 LSU sequence it was reported as Ribotype 1 from Belize (Litaker et al., 2010)

Other specimens examined: Clonal strain VGO1167 isolated from samples obtained in Punta Hidalgo, Tenerife on September 11th, 2013.

METHODS

Source of specimens and culture conditions. Samples of diverse macroalgae were were collected in tidal ponds at La Puntilla (28º 8.9' N, 15º 26'W), in Las Palmas, Canary Islands in February 2010, and in Punta Hidalgo (28º 34'N, 16º 19'W), a rocky shore on the north coast of Tenerife, Canary Islands in September 2013. Due to logistical constraints derived of opportunistic samplings, the area was sampled only during low tide and samples were taken from high level tidal ponds to upper infralitoral zone accessible by simple snorkelling at no more than one meter depth. Samples of small mixed seaweeds were collected from tidal pools on the rocks during low tide, placed in plastic bottles and shaken. Afterwards, the gross materials were removed through a sieve and the remaining seawater was used for cell isolation. Isolation was carried out by a capillary pipette with the aid of a Zeiss Invertoscop D microscope (Carl Zeiss AG, Germany) and isolated cells were incubated in 96 microwells plates in full strength K/2 medium (Keller et al., 1987, Guillard & Hargraves, 1993) made with seawater from Ría de Vigo with a salinity adjusted to 34 and incubated at 24 ºC and a photon irradiance of about 90 µmol $m^{-2} \cdot s^{-1}$ of PAR measured with a QSL-100 irradiameter (Biospherical Instruments Inc. San Diego, CA, USA) and at a 14:10 L:D photoperiod. The cultures are deposited at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo. These and other cultures used in this study obtained from different sources, are listed in Table S1. As no significant differences were observed among wild and cultured cells, the study was based on cultured material.

Light microscopy

The cultured cells were observed alive or fixed with formalin. For plate pattern identification the cells were stained with Fluorescent Brightner 28 (Sigma, St Louis, MO, USA) following a modified Fritz and Triemer (1985) technique. Others were dissected, squashing the cells by pressing the cover slip over them and sometimes with the aid of sodium hypochlorite. The nuclei were stained using SYBR Green (Molecular Probes, Eugene, OR, USA) following the method of Figueroa and Bravo (2005). Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence. The photographs were taken with a Canon EOS D60 (Canon Inc., Tokyo, Japan) or with an Axiocam HRc (Carl Zeiss, Jena, Germany) digital cameras. When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and automatically merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The areas of each layer that were out of focus were erased and the layers flattened to get a final image with an enhanced depth of

field. Cell sizes were measured with a Zeiss Axiovert 125 (Carl Zeiss, Jena, Germany) microscope with a Moticam 1 digital camera (Motic Spain, S.L.U.) ant its software.

Sample preparations for SEM.

Five mL of exponentially growing cultures were fixed with GTA at a final concentration of 2%. After two hours at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH followed by Hexamethyldisilazane . After being air dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a Phillips XL30 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

Nomenclature

In this study, the modified Kofoid (1909b) tabulation nomenclature proposed by Besada et al (1982) was used. This was the system used in the last two descriptions of new species of *Gambierdiscus, G. excentricus* (Fraga et al., 2011) and *G. scabrosus* (Nishimura et al., 2014), because it allows comparisons with other genera of Gonyaulacales based on plates homologies. The terms «length» as apical/antapical distance, «width» as transdiameter and depth for dorso/ventral distance were used for the dimensions.

DNA extraction, PCR amplification and sequencing.

Exponentially growing cultures of *Gambierdiscus* (1mL volume) were harvested by centrifugation (13,000 rpm, 2 min) using a Eppendorf 5424R centrifuge (Eppendorf AG, New York, USA), the cellular pellets were rinsed in 1mL distilled water, centrifuged again and the supernatant discarded. Samples were frozen overnight at -20ºC and DNA extraction was done using a modified Chelex procedure (Richlen & Barber, 2005). 100 ?L of 10% Chelex 100 (Bio-Rad, Hercules, CA, USA) in dH2O was added and samples were transferred to 200 ?l tubes. The samples were boiled at 95ºC in a Surecycler 8800 thermocycler (Agilent Technologies, Santa Clara, CA, USA) for 10 min, then vortexed. The boiling and vortex steps were done twice. Samples were centrifuged (13,000 rpm, 2 min) and the supernatants were transferred to clean 200 ?L tubes avoiding to carryover the Chelex beads. Genomic DNA was quantified and checked for its purity in a Nanodrop Lite spectrophotometer (ThermoScientific, Waltham, MA, USA). When necessary, DNA samples were further purified using a standard ethanol precipitation protocol (Surzycki, 2000) and stored at -20 ºC until further processing.

PCR amplification and DNA sequencing

The D1–D3 and D8–D10 regions of the LSUrRNA gene were amplified using the pairs of primers D1R/LSUB (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3') and FD8/RB (5'-GGATTGGCTCTGAGGGTTGGG-3'/5'-GATAGGAAGAGCCGACATCGA-3') (Litaker et al., 2003, Chinain et al., 1999, Scholin et al., 1994) respectively. Amplification reaction mixtures (25 ?L) contained 4 mM MgCl2, 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, CA, USA), and 1-2 ?L from the Chelex extractions. The DNA was amplified in a Surecycler 8800 thermocycler following the conditions detailed elsewhere (Chinain et al., 1999, Litaker et al., 2003). A 10 ?L aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and GelRed™ nucleic acid gel staining (Biotium, Hayward, CA, USA).

The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Purified DNA was sequenced using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and migrated in an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain). The D1–D3 and D8–D10 sequences obtained in this study were deposited in GenBank (for Acc.Nos. see Table S1 in supplementary data, and Figs. 18 and 19).

Phylogenetic analyses.

LSU sequences were inspected and aligned using CLUSTALW multiple alignment in Geneious® Pro 5.6.6 (Biomatters Ltd.). Net average genetic distances ($dA = d_{XY} - (d_X + d_Y)/2$, where d_{XY} is the average distance between groups X and Y, and d_X and d_Y are the mean within-group distances).) were calculated between *Gambierdiscus* clades for the original alignments using MEGA 6. D1-D3 and D8/10 alignments included 1048 and 816 positions. In the case of D8-D10 alignment, poorly aligned positions and divergent regions were checked using GBLOCKS (Castresana, 2000). A final number of 583 bases (71% of the original positions) were saved by GBLOCKS and used in further genetic analyses. Phylogenetic model selection (ML) was performed on MEGA 6. A K2+G model was selected for the D1-D3 and D8-D10 alignments, with gamma shape parameter = 1,06 and 0,43, respectively.D8-D10 *Gambierdiscus yasumotoi* sequences were used to root the trees. The phylogenetic relationships were determined using bayesian phylogenetic inference and ML settings as specified above. Bayesian trees were performed withMrBayes v3.2,(Huelsenbeck & Ronquist, 2001) and the program parameters were statefreqpr = dirichlet (1,1,1,1), nst = mixed, rates = gamma. The phylogenetic analyses involved two parallel analyses, each with four chains. Starting trees for each chain were selected

randomly using the default values for the MrBayes program. The corresponding number of unique site patterns was 769 and 209 in D1–D3 and D8–D10 analyses. The number of generations used in these analyses was 400,000 . Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization (''burn-in'' phase). All final split frequencies were less than 0.06. Maximum Likelihood phylogenetic analyses were conducted in MEGA 6 (for D1-D3 alignment) and PhyML 3.0 (Guindon *et al.*, 2010) for D8-D10 sequences on the South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml). Bootstrap values were estimated from 1000 replicates. Overall topologies by ML and Bayesian inference method were very similar. The phylogenetic tree was represented using the Bayesian inference with posterior probabibility and bootstrap values from the ML method.

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CAPÍTULO 4

ECOLOGÍA DE DINOFLAGELADOS BENTÓNICOS

REVIEW OF THE MAIN ECOLOGICAL FEATURES AFFECTING BENTHIC DINOFLAGELLATE BLOOMS

ABSTRACT

Both benthic and planktic dinoflagellates can produce harmful algal blooms. However most of the studies conducted so far emphasized on planktic species. In the present review, we assessed the main ecological factors affecting the population dynamics of bloom-forming benthic dinoflagellates, with particular emphasis on *Ostreopsis* and *Gambierdiscus*. Based on the basic equation of population dynamics, we mainly focused on growth, predation, mortality, immigration and dispersion. Factors determining the dynamics of benthic dinoflagellate populations are very different from the well-studied case of planktic dinoflagellates. The relative movement of cells and water is the main difference as benthic dinoflagellates depend on a fixed substratum while planktic dinoflagellates depend on a water body. Any alteration in the substratum will affect benthic dinoflagellate populations, as for example the changes in seaweeds concentrations due to predation by sea urchins. We also evaluated the impact of global changes on dinoflagellates bloom occurrence

INTRODUCTION

Most authors working on harmful algal blooms (HAB) have their main experience in plankton rather than benthos ecology. Recently, the importance of benthic HABs is gaining increasing interest, mainly based on the impact of ciguatera, a syndrome caused by dinoflagellates of genus *Gambierdiscus*, which is the most important food borne disease of non-bacterial origin on the world (Parsons *et al.*, 2012), and on the likely relationship between *Ostreopsis* blooms and respiratory problems in the Mediterranean Sea shores (Mangialajo *et al.*, 2008). These harmful benthic dinoflagellate genera that cause HABs are mainly epiphitic on macroalgae growing on shallow rocky habitats.

The basic equation of population dynamics is:

dN/dt = (Growth + Immigration) - (Predation + Mortality + Dispersion)

Thus, algal blooms dynamics depend on the balance between gains and losses of cells. If gains, due to replication or advection of cells are higher than losses from grazing, mortality and dispersion, a bloom will occur. While these terms have been well studied for phytoplankton, this is not the case for benthic dinoflagellates, which usually grow in a shallow and well-illuminated environment where nutrients are usually scarce or depleted

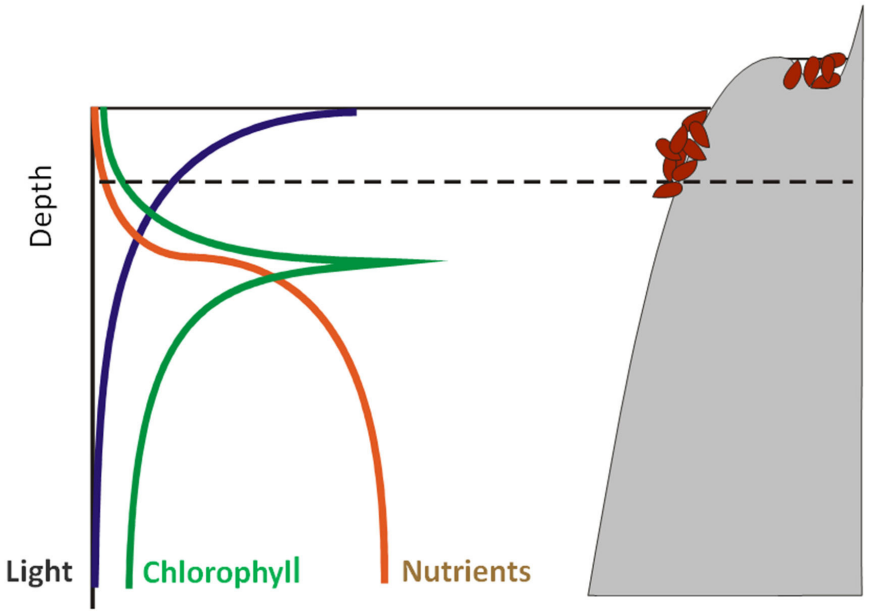

Fig. 1. Warm water benthic dinoflagellates usually grow in nutrient depleted waters with high irradiance, at a lesser depth than the planktic chlorophyll maximum.

(Tindall & Morton, 1998, Shears & Ross, 2009, Pistocchi *et al.*, 2011). Here we review the singularities of the main factors of this equation for benthic HABs in comparison to planktic HABs.

GROWTH

Growth of photoautotrophs depends on the fixation of inorganic carbon and the uptake of nutrients. Carbon fixation depends on light energy through photosynthesis while nutrient uptake is limited by their availability in the seawater, physical constraints and both processes are temperature-dependent. Phytoplankton communities are associated with a particular water body, but benthic dinoflagellates are associated with a substrate (Fig. 1). At a mesoscale, while planktic species play an important role in the water body history where they grow, for example, depleting its nutrients in the photic zone, this is not the case of benthic dinoflagellates which, being linked to a substrate, are affected by water with characteristics defined elsewhere. In addition, benthic species play a minimal role in defining the chemical properties of water bodies, since at a mesoscale their biomass is very small compared to that of phytoplankton for a big water volume.

Light provides energy for carbon fixation through photosynthesis. Benthic dinoflagellates are common in surface pristine waters where light is intense and

photoinhibition can be expected unless they use strategies to avoid high irradiances. For example, Ballantine *et al*. (1988) suggested that *Ostreopsis lenticularis* can migrate to shaded areas of the macroalgae. Using PAM fluorometry, P-E curves (Rapid Light Curves (RLC), of relative Electron Transport Rate (rETR) *vs* irradiance) (Schreiber, 2004) can be obtained within a few minutes in a non invasive way. High-light adapted organisms have higher maximal photosynthetic rates and their photosynthetic apparatus saturates at higher irradiances compared with low-light adapted cells. P-E curves obtained by PAM fluorometry on some benthic dinoflagellates include characteristics more typical of shade-adapted rather than high-light adapted organisms. This is the case of *Gambierdiscus*, which may exploit the three-dimensional structure of the algal host thallus to minimize light exposure (Villareal & Morton, 2002). During a virtually monospecific bloom of *Ostreopsis* cf. *ovata* studied in Llavaneres, NW Mediterranean Sea (unpublished results), P-E curves were obtained at different depths (2, 4 and 7m) with a cuvette version of Walz Water-PAM fluorometer. The photosynthetic parameters in RLC's showed that dinoflagellates from surface and deep samples were light-adapted and shade-adapted, respectively, at a given time during the day. But these RLC's were also variable during a 24h cycle. As a general trend, higher rETR values were observed in the morning reaching a mid-day maximum, and decreasing rETR values from mid-day till a mid-night minimum (Fig. 2).

Another way to study how benthic dinoflagellates are adapted to light is the study of their pigment composition, e.g. the ratio of peridinin to chl c_2 as proposed by Zapata *et al* (submitted). These authors argue that low-light adapted benthic dinoflagellates like *Prorocentrum levis* exhibit higher peridinin to chl c_2 ratios, while planktic species like *Alexandrium tamarense* that can bloom in well illuminated surface waters display lower ratios. *Ostreopsis* and *Gambierdiscus* showed intermediate values (Zapata *et al.*, submitted).

At very low Reynolds numbers, cells live in an environment where viscosity is more important than inertia (Purcell, 1977, Berg & Purcell, 1977). In very turbulent waters the viscosity in the seawater smoothes out fluctuations smaller than a few millimetres (Lazier & Mann, 1989) so nutrient uptake by small cells, less than 100 μm in diameter, will depend only on diffusion. If nutrient concentration in bulk water is low, when nutrient uptake by the cell is higher than the rate of diffusion, a nutrient-depleted zone develops around the cell. Planktic organisms having the ability to swim can reduce to some extent the thickness of this nutrient-depleted zone, thus increasing diffusion towards the cell surface (Kiørboe, 1993). In the benthic environment, as cells are linked to the substrate, their relative movement to water surrounding them depends more on water motion than on swimming. As water movement due to currents or waves can be about three orders of magnitude higher than dinoflagellates swimming speed, the ability of benthic

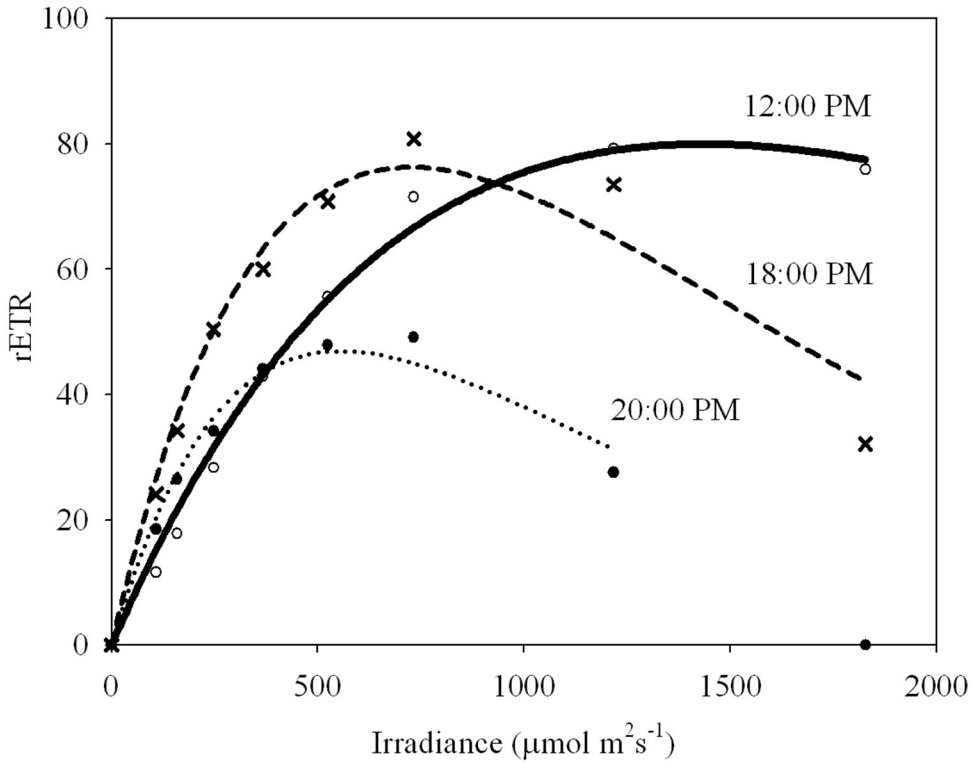

Fig. 2. Fluorescence rapid light curves (RLC) obtained by PAM fluorometry of field samples taken from seaweeds at 0.5 m depth during a bloom of *Ostreopsis* cf. *ovata* in Llavaneres, NW Mediterranean sea during a day

dinoflagellates to take up nutrients in low nutrient waters is higher than that of phytoplankton. The efficiency of benthic species in nutrient uptake depends not only on their own physiological characteristics like V_{max} or K_s, but also on water velocity according to the mass-transfer theory (Atkinson, 2001). As viscosity decreases when temperature increases, high temperatures will favour nutrients transport.

Nutrient remineralisation in sediments is important and also enhanced by warm temperatures, so release of nutrients by the sediments can provide a continuous nutrient supply that can be instantaneously taken up by all benthic algae and hence, nutrient concentrations remain low. All these factors may explain why benthic dinoflagellates can bloom in warm and nutrient poor waters (Tindall & Morton, 1998, Shears & Ross, 2009, Pistocchi *et al.*, 2011).

There is no clear-cut limit to differentiate benthic from planktic dinoflagellates, so a continuum from planktic to benthic behaviour can be found and hence, many of their associated characteristics show also a continuum. Benthic forms are mainly epiphytic and tend to be flatter than those which are more planktic. Being flattened, the surface/volume ratio is higher than in spherical cells, so this shape helps nutrient uptake under oligotrophic conditions. *Ostreopsis* is flatter and more benthic than *Coolia*, and also prefers more stirred waters compared with *Coolia* (Vila *et al.*, 2001).

The f/2 medium is the most commonly used to grow marine microalgae giving excellent results in most cases. However, there are many experiences in which, using full strength medium to grow benthic dinoflagellates, the cells appear distorted and they do not grow well, while those grown on a diluted medium look much healthier (Holmes *et al.*, 1990). This can reflect the fact that they are adapted to grow in low nutrient environments (Tindall & Morton, 1998, Shears & Ross, 2009, Pistocchi *et al.*, 2011).

In addition to being well adapted to oligotrophic conditions, mixotrophy cannot be discarded. The presence of red bodies in many cells of *Coolia* or *Ostreopsis* is frequently reported (Aligizaki & Nikolaidis, 2006, Selina & Orlova, 2010) and could represent an effect of mixotrophy although this has not been proven. These bodies were observed only in field samples, but when the cells with these bodies are isolated and grown in artificial inorganic medium, the bodies disappear (Authors personal observations).

The importance of temperature for the development of benthic HABs is reflected in the fact that these events occur mainly in tropical waters or in temperate areas during summer (Pistocchi *et al.*, 2011, Parsons et al., 2012). *Ostreopsis* is considered a tropical or subtropical species, but records in colder waters are increasing. It has been observed in temperate regions at latitudes higher than 40º such as the Basque coast of Spain (Laza-Martinez *et al.*, 2011), near Vladivostok, Russia (Selina & Orlova, 2010) and Wellington, New Zealand (Rhodes, 2011). In the Mediterranean Sea, *Ostreopsis* blooms in summer (Mangialajo *et al.*, 2011, Vila et al., 2001) when nutrients are relatively low and light and temperature are high. In all cases, it is clear that when *Ostreopsis* blooms the water is warm although in some cases the bloom may occur after the temperature maximum (Selina & Orlova, 2010, Totti *et al.*, 2010). High water temperatures, high irradiance and high remineralisation are factors that converge to create an environment favouring benthic HABs.

PREDATION

Little is known about the predators able to control populations of benthic dinoflagellates, although the role of invertebrates might be important. Nevertheless it is well known that ciguatera is a syndrome caused by toxins produced by epiphytic

dinoflagellates of genus *Gambierdiscus* which are accumulated in fish through the food web. Herbivorous fish are the first predators of *Gambierdiscus* when grazing macroalgae eating also the epiphytes. In this case fish not only predate the dinoflagellates but also their substrate (i.e. seaweeds) limiting further development of the bloom.

MORTALITY

The role of parasites such as perkinsid (Alveolata) is being increasingly recognized in planktic species (Norén *et al.*, 1999, Figueroa *et al.*, 2008) and they also affect benthic species (Leander & Hoppenrath, 2008). They have a zooid stage that can infect dinoflagellate cells where they multiply to form a spherical sporangium that liberates many new zooids. They have been tested in many cultures and it has been observed that they can infect many different species. Parasite zooids are abundant when there is a bloom of a susceptible species and they can affect the concentration and even cause the end of a bloom, nevertheless it is unlikely that they control the growth dynamics of the host species when the latter is in low abundance.

IMMIGRATION AND DISPERSION

Immigration and dispersion are very important factors in planktic blooms but as benthic dinoflagellates are more or less attached to the substrate, their circumstances are quite different. Benthic dinoflagellates use different mechanisms for being benthic. *Prorocentrum levis* or *Prorocentrum lima* are strongly attached to the substrate so their dispersion is difficult. Other taxa, like *Ostreopsis*, are attached by mucus threads that form a kind of spider web which encloses the cells and whose size increases as the cells multiply (Fraga unpublished observations). The production of mucus by *O. lenticularis* is higher at higher irradiances (Heil *et al.*, 1993) and this species also forms gas bubbles from daylight photosynthesis. Together, these factors allow this species to float and detach from the substrate, drift to the surface and colonize other areas, helped by the usually stronger winds during the day. Exposure to wind and wave action explains differences in distribution due to dispersion (Shears & Ross, 2009).

Almost nothing is known about the life strategies of benthic dinoflagellates. For instance, it is unclear whether these organisms have particular life-cycle features as a consequence of their different ecological positions compared to planktic species. Few cyst descriptions have been published. Only short-term pellicle cysts from sexual reproduction in *Coolia monotis* (Faust, 1992) and pellicle and thecate cysts of *O.* cf *ovata* (Bravo *et al.*, 2012) have been described. In turn, no dormant resting cysts were detected neither in culture nor in nature. The fact that pellicle and thecate cysts of *O.* cf *ovata* from bloom-incubated samples were able to germinate up to 6 months after their

formation suggests that those cysts may constitute an overwintering population responsible for bloom recurrence (Bravo *et al*., 2012).

GLOBAL CHANGE IMPACT

During the last decade, reports of respiratory problems among people who were in some places of the Mediterranean coast have created public concern in the affected areas. Due to circumstantial evidence, these problems have been related to blooms of the dinoflagellate *Ostreopsis* (Rossini, 2011). Although an increasing awareness of these problems is evident, it is possible that the increase in *Ostreopsis* bloom events has been caused by a phase shift in benthic microalgae communities due to anthropogenic or natural environmental change. These reports of intoxications become common in the Mediterranean Sea after mass mortalities of benthic invertebrates were recorded as a consequence of the exceptional 2003 heat wave (Garrabou *et al.*, 2009). The mortalities could also affect non identified potential predators of *Ostreopsis*, and a potential cascade effect leading to an increase in *Ostreopsis* populations.

Long-term phytoplankton population changes have been reported (Edwards *et al.*, 2006, Beaugrand *et al.*, 2008) but they were smooth and no catastrophic phase shifts were observed in phytoplankton over inter-annual scales. Nevertheless, benthic dinoflagellates live in a more complex and fragile system. Abrupt and catastrophic phase shifts from coral to macroalgal domination have been reported in coral reefs (Hughes, 1994, Bellwood *et al.*, 2004, Nyström *et al.*, 2000) where benthic dinoflagellates are common. The impact of these ecosystem shifts on the populations of benthic dinoflagellates have not been studied in depth, but, for example, we could expect that when a system dominated by corals shifts to one dominated by macroalgae (Hughes, 1994), a change in dinoflagellate communities may also occur.

It is known that sea urchin populations play a key role in the ecology of shallow rocky systems (Lawrence, 1975). In temperate kelp forests, an increase in the abundance of sea urchins due to, for example, a grazing reduction, may create barren zones (Shears & Babcock, 2003, Jackson *et al.*, 2001) whereas in tropical coral reefs a reduction in the populations of sea urchins may cause a shift from a coral dominated to macroalgae dominated system (Hughes, 1994). Increases in sea urchin abundance may even favour the colonization of invasive coral species in areas that were previously dominated by macroalgae (Coma *et al.*, 2011). Obviously, changes in the benthic communities, in particular macroalgal abundance, are likely to play a determinant role in the dynamics of epiphytic dinoflagellates success. Therefore, a close relation between *Ostreopsis* and sea urchins population dynamics is likely. While *Ostreopsis* blooms may kill or severely damage sea urchins (Vila et al., 2008, Ferreira, 2006, Shears & Ross, 2009, Sansoni *et*

al., 2003), the latter can destroy the habitat of *Ostreopsis* by removing macroalgae from rocky substrates (Shears & Ross, 2010) so a balance between sea urchins and *Ostreopsis* might be important.

Another common alteration of reefs is coral bleaching caused by high sea surface temperatures (Glynn, 1993), which has already been associated to ciguatera outbreaks caused by overgrowth of *Gambierdiscus* (Hallegraeff, 2010, Bagnis *et al.*, 1992) as an opportunistic species.

CONCLUSIONS

The main ecological difference between benthic and planktic dinoflagellates is that while planktic species depend on a water body, benthic species depend on a substrate. Hence, some of the well studied processes in plankton dynamics of harmful algal blooms are not applicable to the study of benthic algal blooms, and hence different approaches to their study should be carried out. Benthic systems may suffer abrupt alterations due to cascade effects triggered by the action of some benthic organisms and/or environmental changes that affect the substrate and trophic relations among species.

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CONCLUSIONES

1. El dinoflagelado tóxico *Gymnodinium catenatum* fue uno de los primeros dinoflagelados desnudos formadores de cadenas en ser descrito. Como consecuencia de esto, en varias ocasiones en que se ha observado un dinoflagelado desnudo formador de cadenas, éste fue citado en la literatura como *G. catenatum*. En esta tesis, como resultado de un estudio detallado de *G. catenatum* y de otros dinoflagelados desnudos, se ha descrito una nueva especie de dinoflagelado, *Gyrodinium impudicum*. Esta especie forma cadenas como *G. catenatum* pero es de menor tamaño, no es tóxica, y tiene una silueta, composición de esteroles y comportamiento diferente. (Posteriormente, en el marco de una redescripción de los géneros *Gyrodinium* y *Gymnodinium* realizada por otros autores, *G. impudicum* fue transferido al género *Gymnodinium*).

2. *Coolia monotis* es un dinoflagelado tecado de hábitos principalmente bentónicos que fue descrito en 1927 de una forma muy elemental, propia de la época. En esta tesis se ha hecho un estudio detallado de cepas en cultivo de *C. monotis* de cerca de la localidad tipo en el Mar del Norte, así como de Galicia y el Mediterráneo y se ha comparado con cepas de Canarias. Como consecuencia se describió una nueva especie de este género, *Coolia canariensis.* La comparación de estas cepas con otras del Mar Caribe, de Indonesia, de Australia y de Malasia permitió la redescripción de *Coolia tropicalis* que originalmente había sido mal descrita.

3. Coincidiendo con el primer caso de ciguatera reportado en la Islas Canarias se comenzó un estudio de los dinoflagelados bentónicos de estas islas. La ciguatera es un envenenamiento propio de zonas tropicales causado por la ingestión de pescado portador de toxinas producidas por dinoflagelados del género *Gambierdiscus.* Sorprendentemente se ha encontrado en Canarias una gran diversidad de especies de este género, que dió lugar a la descripción de dos especies nuevas, *G. excentricus* y *G. silvae*, y a la primera cita en el Océano Atlántico de *G. australes* que anteriomente sólo se conocía en islas del Océno Pacífico.

4. La principal diferencia ecológica entre los dinoflagelados planctónicos y los bentónicos es que, mientras los primeros están asociados a un determinado cuerpo de agua sujeto a una evolución, los segundos al estar fijados a un sustrato inmóvil, están continuamente cambiando de cuerpo de agua. El movimiento relativo de las células con respecto al agua es, en el caso de los bentónicos, de varios ordenes de magnitud mayor que el de los planctónicos.

5. Los problemas causados por las microalgas nocivas hacen que se profundice en el estudio de esas especies para minimizar sus efectos. Este hecho frecuentemente lleva al

descubrimiento de nuevas especies que anteriormente se confundían con otras. Es fácil deducir que si se pone el mismo esfuerzo en estudiar especies que no son sospechosas de producir daños, que aquel que se pone en estudiar las nocivas, se descubrirán numerosas especies nuevas.

6. En muchos casos existe un conflicto entre la alfataxonomía clásica, basada en morfología, y los estudios genéticos. Es necesario revisar numerosos géneros para poner de acuerdo ambas visiones de un mismo problema.

7. Hay que tener especial cuidado con la identificación de especies en un determinado lugar ya que es frecuente la coexistencia de especies congenéricas pseudocrípticas.

8. Al igual que puede haber diferencias notables en la toxicidad de especies pseudocrípticas, también puede haber otras de comportamiento o fisiológicas que afecten a su nicho ecológico. Una identificación incorrecta de una especie puede dar lugar a conclusiones ecológicas equivocadas.

9. En la descripción de nuevas especies es indispensable utilizar más criterios que el morfológico, incluyendo al menos, además, la secuenciación genética de uno o más genes. La determinación de algunas características fenotípicas bioquímicas tales como pigmentos, esteroles o toxinas también puede ayudar en la caracterización de las especies.

RESUMEN

INTRODUCCIÓN

En este capítulo se hace una revisión sobre los conceptos básicos utilizados en taxonomía y en los distintos conceptos de especie con especial énfasis en los dinoflagelados. Se aborda la necesidad de la taxonomía para estudios ecológicos y de biodiversidad. Se describe el desarrollo histórico de la taxonomía y los distintos conceptos de especie utilizados en los estudios de dinoflagelados. Se definen especies crípticas, semicrípticas y pseudo-crípticas y se revisan las propuestas taxonómicas actuales para resolver el problema de las especies crípticas y su caracterización, entre ellas la llamada taxonomía integradora que incluye más de un tipo de caracteres para la definición de especies.

Se describen las principales características de los dinoflagelados, y se revisan los dinoflagelados nocivos en base al tipo de daños que éstos pueden producir.

Dentro de la descripción de los criterios utilizados para taxonomía de dinoflagelados, se propone un sistema de nomenclatura de las placas de dinoflagelados tecados algo diferente al habitual utilizado por otros investigadores pero que permite la comparación de especies de diferentes géneros de Gonyaulacales en base a la homología de las placas de sus tecas.

CAPÍTULO 1. GÉNERO *GYMNODINIUM*

En este capítulo se tratan dos especies de *Gymnodinium* que durante muchos años no se diferenciaron como como dos especies distintas: El tóxico *G. catenatum* y el no tóxico *G. impudicum*. La segunda fue confundida en varias ocasiones con la primera dando lugar a falsas alarmas hasta que fue descrita como una especie nueva.

Este capítulo está basado principalmente en los siguientes trabajos publicados y en información inédita:

Estrada, M., F.J. Sánchez y S. Fraga. (1984). *Gymnodinium catenatum* Graham en las rías gallegas (NO de España). Investigación Pesquera, 48(1): 31-40. (Constituye la primera cita de *G. catenatum* en el Océano Atlántico Norte).

RESUMEN

Fraga, S., I. Bravo, M. Delgado, J.M. Franco, y M. Zapata (1995). Differences between two chain forming, athecate, red tide dinoflagellates: *Gymnodinium catenatum* Graham and *Gyrodinium* sp. En: Harmful Marine Algal Blooms. Lassus et al. eds. Lavoisier, París, pp.39-44. (Se reporta la posible existencia de dos especies pseudocrípticas diferentes).

Fraga, S., I. Bravo, M. Delgado, J.M. Franco y M. Zapata (1995). *Gyrodinium impudicum* sp. nov. (Dinophyceae), a non toxic, catenate, red tide dinoflagellate. Phycologia, 34(6): 514-521. (Se describe una nueva especie diferente de *G. catenatum*).

Hasta hace pocos años, la mayoría de los dinoflagelados desnudos estaban adscritos al orden Gymnodiniales, y dentro de este orden, el género *Gymnodinium* era y es el que más especies comprende. Con el advenimiento de las técnicas moleculares y la mejora de las de microscopía electrónica se están erigiendo continuamente nuevos géneros a partir de especies que anteriormente estaban en el género *Gymnodinium*. Los principales géneros de este orden se diferenciaban por unos pocos caracteres morfológicos tales como la situación del cíngulo en relación al eje longitudinal, si éste tenía o no desplazamiento cingular, o si daba más de una vuelta a la célula. De este modo, antes del año 2000, el género *Gymnodinium* se diferenciaba de *Gyrodinium* en si el desplazamiento cingular era menor o mayor de un quinto del la longitud total de la célula. Siguiendo estos criterios se describió en 1943 *Gymnodinium catenatum* Graham en el Golfo de California donde formaba una conspícua marea roja sin que en ese momento se tuviesen noticias de su posible toxicidad. Después de dos citas aisladas, una en Argentina y otra en Japón, en 1976 esta especie se observó en elevadas concentraciones en Galicia coincidiendo con intoxicaciones de tipo paralizante que causaron numerosos intoxicados por mejillón gallego en varios países europeos. En años posteriores volvió a observarse y eso permitió su aislamiento y cultivo proporcionando material para su estudio. *G. catenatum* es un dinoflagelado desnudo y fotosintético de 31-39 ìm de largo por 37-42 ìm de ancho, formador de cadenas, con un cierto desplazamiento y entrecruzamiento cingular. En esas primeras observaciones en Galicia se observaron células de un tamaño menor e incluso llegó a aislarse una cepa que no era tóxica y mostraba un comportamiento diferente al de las típicas células de *G. catenatum*. Como consecuencia de una marea roja en el puerto de Valencia que, en un principio se consideró que era de *G. catenatum*, se aislaron nuevas cepas que eran iguales a la que se consideraba como una cepa anormal de G. catenatum de Vigo y permitieron la descripción de una nueva especie para la ciencia, *Gyrodinium impudicum* Fraga et Bravo.

Para la descripción de esta nueva especie se utilizaron cultivos tanto obtenidos a partir del aislamiento con micropipeta de cadenas móviles del plancton como a partir de incubación de fango con, presumiblemente, quistes que no llegaron a identificarse en ese

momento. Las células se observaron vivas con microscopía de luz y fijadas con glutaraldehido y tetróxido de osmio para observación al microscopio electrónico de barrido.

G. impudicum es un dinoflagelado fotosintético desnudo de 14-37 µm de largo y 16 a 32 µm de ancho. Típicamente, forma cadenas de 4 células, aunque se han visto cadenas más largas y más cortas o células solitarias. El núcleo es central, pero ligeramente desplazado hacia el hipocono en la célula anterior de una cadena, y hacia el epicono en la posterior. El cíngulo es profundo, con un desplazamiento entre 1/3 y 1/4 de la longitud total de la célula. El surco es estrecho y penetra en el epicono hasta el ápice, donde gira en sentido antihorario visto desde el ápice, para formar una acrobase profunda.

Esta especie había sido confundida con *G. catenatum* en algunas publicaciones en el Mediterráneo o incluso con *Polykrikos* en Japón. Probablemente haya sido también confundida con *Cochlodinium* formadores de cadenas.

En resumen, las principales diferencias entre *G. catenatum* y *G. impudicum*, dos especies que previamente podrían considerarse como crípticas son: El tamaño medio, la longitud de la cadena, el desplazamiento cingular, la forma de la acrobase, la silueta del hipocono, la forma y número de los cloroplastos, la toxicidad, el comportamiento natatorio, la producción de moco y la composición de esteroles.

Estudios genéticos posteriores realizados por otros autores demostraron que estas dos especies son claramente diferentes genéticamente. Una vez redescritos los géneros *Gymnodinium* y *Gyrodinium* en el año 2000, *G. impudicum* fue transferido al género *Gymnodinium*.

CAPÍTULO 2. GÉNERO *COOLIA*

En este capítulo se tratan varias especies del género *Coolia* que en algunos casos han sido consideradas de forma errónea como *C. monotis*. Se describe una nueva especie, *Coolia canariensis*, y se redescribe *Coolia tropicalis*. Se asigna a esta última especie una cepa tóxica australiana reportada como *C. monotis* y que ha dado lugar a la consideración de *C. monotis* como una especie tóxica cuando en realidad no lo es.

Este capítulo está basado principalmente en los siguientes trabajos publicados y en información inédita:

Fraga, S., Penna, A., Bianconi, I., Paz, B. & Zapata, M. 2008. *Coolia canariensis* sp. nov. (Dinophyceae), a new nontoxic epiphytic bentic dinoflagellate from the Canary Islands. J Phycol 44:1060-70. (Se describe una nueva especie de *Coolia*. En la versión publicada de este artículo se utilizó la nomenclatura de las placas utilizada en aquel

momento. En esta memoria, esa nomenclatura ha sido corregida siguiendo los criterios expuestos en la introducción).

Mohammad-Noor, N., Moestrup, Ø., Lundholm, N., Fraga, S., Adam, A., Holmes, M. J. & Saleh, E. 2013. Autecology and phylogeny of *Coolia tropicalis* and *Coolia malayensis* (Dinophyceae), with emphasis on taxonomy of *Coolia tropicalis* based on light microscopy, scanning electron microscopy and LSU rDNA. J Phycol 49:536–45. (Se redescribe *Coolia tropicalis* cuya descripción original de Maria Faust se consideró que era errónea).

Se describe una nueva especie de dinoflagelado fotosintético, *Coolia canariensis* Fraga, a partir de muestras tomadas charcas de marea en la costa rocosa de las Islas Canarias. Su morfología se estudió mediante microscopía óptica y electrónica de barrido. Las células con casi esféricas y tienen una teca lisa y gruesa con muchos poros dispersos. La placa 4' es la mayor de las de la epiteca y la 6" es el doble de ancha que larga. La filogenia inferida a partir de las regiones D1/D2 de la subunidad grande del ADN ribosómico nuclear de tres cepas de *C. canariensis* y de varias cepas de otras especies de *Coolia, C. monotis* y *C.* sp. muestran que las cepas de *C. canariensis* se agrupan en un clado distinto de los de las otras especies. No se detectaron toxinas meidante el bioensayo de ratón, LC-FLD o LC-MS. Su composición pigmentaria es del tipo de dinoflagelados con peridinina. En este trabajo se aumenta el número de especies de este género y ayuda a definir mejor la especie tipo del género, *Coolia monotis* Meunier. Junto a esta nueva especie, se han analizado muchas otras cepas de *C. monotis* del Océano Atlántico y del mar Mediterráneo en busca de toxinas y no hay evidencia de la producción de toxinas relacionadas con la YTX (yesotoxinas) en ninguna de ellas, como se sugirió anteriormente para *C. monotis* de Australia.

En el trabajo en el que se describe la toxicidad de *C. monotis* en Australia se muestra una foto en que la placa 6" es muy ancha en comparación con las de *C. monotis* y *C. canariensis* mostrando que claramente no se trata de ninguna de esas dos especies. En células de una cepa aislada de una muestra tomada en Manado, Indonesia, al sur del Mar de las Célebes, y en otra aislada de Belize y obtenida de la colección del NCMA (Antígua CCMP) se observó que esta placa era igual a la de la foto de la cepa australiana y a la de la descripción de *Coolia tropicalis* Faust. Sin embargo, la forma de las demás placas de la hipoteca era diferente de las descritas por Faust. Solicitadas más fotos de microscopía electrónica al autor del trabajo autraliano, se observó que coincidían morfológicamente con las cepas de Manado y de Belize, ésta última tomada en la localidad tipo de *C. tropicalis*. Dado que en la descripción original de esa especie no se observa ninguna foto de todas la placas epitecales y que las diferencias eran con un dibujo, se redescribió *C. tropicalis* en base a que la cepa de Belize era de la localidad tipo y que la forma de las cepas de Indonesia y otras de Malasia coincidian con la australiana y con todos los detalles

observables en las fotos de la descripción original de *C. tropicalis* pero no con los dibujos. Principalmente se corrigió la forma de la placa 4' que en lugar de tener forma de cuña como en la descripción original, es casi rectangular.

CAPÍTULO 3. GÉNERO *GAMBIERDISCUS*

En este capítulo se tratan tres especies de *Gambierdiscus* encontradas en las Islas Canarias. Se describen dos nuevas especies, *G. excentricus* y *G. silvae*, y se reporta por primera vez en el Océano Atlántico la presencia de *G. australes*. Este capítulo está basado principalmente en los siguientes trabajos publicados y en información inédita:

Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N. & Zapata, M. 2011. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean) Harmful Algae 11:10-22. (Descripción como nueva especie, de *Gambierdiscus excentricus*).

Fraga, S. & Rodríguez, F. 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. (Enviado a PROTIST) (Descripción como nueva especie, de *Gambierdiscus silvae*, y primera cita de *G. australes* en el Atlántico).

Se describe *Gambierdiscus excentricus* Fraga et Rodríguez, un nuevo dinoflagelado tóxico bentónico de las Islas Canarias. Se aisló a partir de muestras de macroalgas que crecen en charcas de marea y su presencia se observó en invierno y en verano. Su morfología se estudió por medio de la microscopía de luz y por microscopía electrónica de barrido (SEM); *G. excentricus* tiene forma lenticular de 83 ± 10 (69-110) µm de ancho, 37 ± 3 (34-41) µm de largo y profundidad media de 97±8 (84 a 115) µm. La fórmula tecal es: Po, 4', 0a, 6'', 6c, ?s, 5''', 0p , 2''''. La placas son lisas con poros circulares u ovalados distribuidos uniformemente. La placa del poro apical Po es ovalada, con una hendidura en forma de anzuelo y está deplazada ventralmente en relación a otras especies de *Gambierediscus*. La primera placa apical, 1' es muy pequeña. La segunda apical 2' es la mayor de la epiteca y tiene la sutura 2'/3' aproximadamene el doble de larga que la sutura 2'/4' . Las placas 1' y 6'' son muy pequeñas y orientadas hacia la parte posterior de la célula debido a la torsión de la zona flagelar que forma como un embudo desde el cual emergen los dos flagelos, de los cuales, el longitudinal se proyecta perpendicularmente. La placa sulcal posterior S.p. está en la hypotheca, fuera del surco longitudinal. La placa 2'''' es dos veces más larga que ancha. El núcleo tiene forma de arco y se encuentra en la parte dorsal de la célula con las puntas orientadas hacia el lado ventral de la célulaLos árboles filogenéticos obtenidos de secuencias de genes de ARN ribosomal de subunidad grande (LSU) muestran una topología que confirma que *G. excentricus* se agrupa en su grupo propio, separado del resto de las especies *Gambierdiscus* y con *G. australes* como

su pariente más cercano. Las células son fotosintéticas y la composición de pigmentos estudiados en cultivos de *G. excentricus*, incluye la peridinina, como el principal carotenoide, la clorofila a y las clorofilas accesorias c_1 y c_2. Los ensayos basados en células de neuroblastoma para ciguatoxinas (CTX) y para maitotoxina (MTX) confirmaron a *G. excentricus* como productor de compuestos del tipo de CTX y MTX. El hallazgo de una especie tóxica de *Gambierdiscus* en Canarias podría explicar los recientes casos de ciguatera resitrados en la zona.

La segunda especie encontrada en aguas canarias es *Gambierdiscus silvae* Fraga et Rodríguez sp. nov.. Su morfología se estudió mediante microscopía óptica y electrónica de barrido. Las células de *G. silvae* están comprimidas en sentido anteroposterior, tienen forma lenticular con la epiteca más alta que la hipoteca y son simétricas en vista ventral. Tienen 46±5 µm de largo, 64±9 µm de ancho y 69±8 µm de profundidad. Son circulares en vista apical y antapicale y tienen la teca gruesa y lisa con muchos poros dispersos. Las placas apicales y antiapicales son más lisas que las placas pre y postcingulares que muestran una depresiones superficiales más visibles cerca del cíngulo. La fórmula tecal es Po, 4', 0a, 6'', 6c, ?S, 5''', 0p, 2''''. La placa del poro apical, Po es ovalada, tiene una hendidura en forma de anzuelo, y está centrada en la epiteca, sólo ligeramente desplazada hacia la parte ventral. La placa 1' tiene forma de punta de flecha, es muy pequeña y no está en contacto con la Po. La placa 2' tiene forma de hacha apuntada, y por lo general, es la mayor de las placas de la epiteca. En cultivos viejos, en los que se observaron parejas de células en fusión, se observaron muchas células muy altas, casi esféricas, y con las placas de pre y postcingulares alargadas en el eje apical, mientras que las placas apicales y antapicales tienen el mismo tamaño que las de las células más corrientes. En la mayoría de las células, las suturas 2'/1'' y 2'/2'' no forman un ángulo, sino una curva continua dándole a la placa 2' la forma de una hacha afilada. La sutura 2'/3'' es aproximadamente el doble de la 2'/1''. La palca 3' es aproximadamente del mismo tamaño que la 4'. Las placas precingulares tienen diferentes tamaños, pero la 2'' es la más amplia y puede ser la mayor de la epiteca en las células altas. La placa 3'' es asimétrica y tiene la sutura 3''/2" más larga que 3''/3'. La placa 6'' es muy pequeña , y con la 1', forma el borde anterior del surco. Las placas 1' , 4' y 1'' no forman un pliegue como el descrito en la literatura para *G. polynesiensis*. El cíngulo es estrecho y profundamente excavado. El surco es como un embudo con el flagelo longitudinal orientado radialmente en el plano ecuatorial en lugar de estar orientado posteriormente como en la mayoría de los dinoflagelados. En la hipoteca, la placa 2'''' es muy amplia y por lo general ocupa la mayor parte de la hipoteca de tal modo que todas las otras placas aparecen como comprimidas en la periferia de la hipoteca. Sin embargo, en células altas el tamaño relativo de 2'''' es más pequeño en comparación como lo es en las células más frecuentes. La placa 1''' por lo general no hace contacto con 2'''', pero a veces estas dos placas más la 1'''' y la 2''' se reúnen en un punto. De una

manera similar, la 2'''' por lo general no está en contacto con la 5''' pero en algunos casos estas dos placas tienen una sutura común, y la placa 2'''' tiene seis lados en lugar de cinco.

La morfología de *G. silvae* no difiere de la descrita por Estela Sousa e Silva para células de las Islas de Cabo Verde reportadas como *Gonidoma* sp. Por esta razón se considera que muy probablemente la especie descrita por Silva sea *G. silvae* y por esta razón se le dedica a esta investigadora portuguesa que fue la primera en describir un *Gambierdiscus* treinta años antes de la descripción del género. En base a las secuencias de la región D8-D10 del la subunidad grande del ADNr, las secuencias del denominado Ribotipo 1 por Litaker et al 2009 del Mar Caribe corresponden a G. silvae.

En las muestras de Canarias también se observó *Gambierdiscus australes* siendo éste el primer registro de esta especie en el Atlántico. Las características morfológicas de las células de *G. australes* coinciden con las descritas para la especie, a saber, la placa 2' de forma rectangular, la placa 2'''' estrecha, la 3'' asimétrica y la superficie lisa de la célula. Las células de *G. australes* de Tenerife tienen una longitud media de 46±6 µm, un ancho de 76±7 µm y una profundidad de 85±8 µm.

Hasta ahora *G. australes* había sido reportado únicamente de las islas del Océano Pacífico. Este trabajo aumenta el número de especies descritas del género *Gambierdiscus*, y además muestra una inesperada diversidad de este género en las Islas Canarias.

Las tres especies de *Gambierdiscus* hasta ahora observadas en Canarias tienen tres patrones biogeográficos diferentes. Mientra *G. excentricus* únicamente ha sido observado en el Atlántico (Canarias, Marruecos y Brasil) excluyendo el Mar Caribe, *G. australes* ha sido observado tanto en le Océano Pacífico como en el Atlántico y *G. silvae* en el Caribe y Canarias. La escasez de trabajos sobre este género en el Atlántico hace que estos datos sean muy preliminares todavía.

CAPÍTULO 5. ECOLOGÍA DE DINOFLAGELADOS BENTÓNICOS

En este capítulo se hace una revisión de los principales factores que determinan las proliferaciones de dinoflagelados bentónicos tomando como referncia a los dinoflagelados planctónicos.

Está basado principalmente en el siguiente trabajo publicado, en dos presentaciones orales en congresos y en información inédita:

Fraga, S., Rodríguez, F., Bravo, I., Zapata, M. & Marañón, E. 2012. Review of the Main Ecological Features Affecting Benthic Dinoflagellate Blooms. Cryptogamie, Algologie 33:171-79.

Fraga, S., Rodríguez, F., Bravo, I., & Zapata, M. 2010. What is different in benthic vs. planktic HABs?. Comunicación oral invitada en el GEOHAB Open Science Meeting on HABs in Benthic Systems patrocinado por el Scientific Committee on Oceanic Research (SCOR) y la Comisión Oceanográfica Intergubernamental (COI) de UNESCO, celebrado en Honolulu, Hawaii, USA del 21 al 23 de junio de 2010.

Fraga, S., Rodríguez, F., Bravo , I. & Zapata, M. 2011. An approach to the study of bloom dynamics of *Ostreopsis*. Comunicación oral invitada en la International Conference on *Ostreopsis* Development. Villefranche-sur-mer, Francia, del 4 al 8 de abril de 2011.

Tanto los dinoflagelados bentónicos como los planctónicos pueden producir floraciones algales nocivas. Sin embargo, la mayoría de los estudios ecológicos realizados hasta el momento enfatizaron sobre especies planctónicas. En la presente revisión, se evaluaron los principales factores ecológicos que afectan a la dinámica de poblaciones de dinoflagelados bentónicos susceptibles de formar floraciones algales nocivas, con especial énfasis en los géneros *Ostreopsis* y *Gambierdiscus*. Con base en la ecuación básica de la dinámica de la poblaciónes, este trabajo se centró principalmente en el crecimiento, la depredación, la mortalidad, la inmigración y la dispersión de las células. Los factores que determinan la dinámica de las poblaciones de dinoflagelados bentónicos son muy diferentes del caso bien estudiado de los dinoflagelados planctónicos . El movimiento relativo de las células y el agua es la principal diferencia ya que los dinoflagelados bentónicos dependen de un sustrato fijo mientras el agua se mueve, y los dinoflagelados planctónicos dependen de un cuerpo de agua con el que ellos se mueven. Cualquier alteración en el sustrato afectará a las poblaciones de dinoflagelados bentónicos, como por ejemplo, los cambios en las concentraciones de algas debido a la depredación de los erizos de mar. Por lo tanto, algunos de los procesos bien estudiados en la dinámica del plancton de las floraciones de algas nocivas no son aplicables al estudio de la proliferación de algas bentónicas, y por lo tanto se deben llevar a cabo aproximaciones diferentes para su estudio. Sistemas bentónicos pueden sufrir alteraciones bruscas debido a los efectos de cascada provocados por la acción de algunos organismos bentónicos y/o cambios ambientales que afectan el sustrato y las relaciones tróficas entre especies. También se evaluó el impacto del cambios global en la ocurrencia de floraciones de dinoflagelados.